

**ROLE OF MICROBIAL
MANGANESE RESPIRATION IN THE
ANAEROBIC CYCLING OF NITROGEN**

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The Academic Faculty

by

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ROLE OF MICROBIAL MANGANESE RESPIRATION IN THE ANAEROBIC CYCLING OF NITROGEN

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LIST OF SYMBOLS AND ABBREVIATIONS

DNA	Deoxyribose nucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
LB	Luria-Bertani growth medium
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RNA	Ribose nucleic acid
<i>DMRB</i>	Dissimilatory metal-reducing bacteria
<i>S. oneidensis</i>	<i>Shewanella oneidensis</i> strain MR-1
Mn	Manganese
DMSO	Dimethyl sulfoxide
TMAO	Trimethylamine oxide
qPCR	Quantitative polymerase chain reaction

SUMMARY

Oxidized forms of manganese (Mn) are strong adsorbants and oxidants of many organic and inorganic compounds. Microorganisms accelerate Mn redox transformations by orders of magnitude and, consequently, greatly influence the biogeochemical fluxes of other metals, carbon, and many trace elements in the environment. Dissimilatory metal-reducing bacteria (DMRB) such as *Shewanella oneidensis* MR-1 reduce iron (Fe) and Mn oxides that are found in sparingly soluble amorphous or crystalline forms. Despite the environmental significance of microbial Mn reduction, the molecular mechanism of microbial Mn respiration remains poorly understood. Soluble Mn(III), although traditionally neglected as an environmentally important oxidant, has been recently found to be a dominant soluble Mn species in aquatic systems. While microbial Mn(III) transformations may represent a major yet overlooked redox process in marine and freshwater systems, little is known about the identity of microbial populations catalyzing Mn(III) reduction in the environment. Furthermore, in situ geochemical analyses indicate a potential microbial link between anaerobic ammonia oxidation and Mn reduction.

The overall aim of the present study was to determine the electron transfer mechanism for Mn(IV) reduction, to identify genes required for anaerobic Mn(III) respiration by *S. oneidensis* MR-1, and to identify microbial consortia that couple NH_4^+ oxidation to Mn reduction in the environment. To meet this overall aim, the specific objectives of the present thesis were to i) generate Mn(III) reduction-deficient mutant strains and identify genes involved in Mn(III) reduction (Chapter 2), ii) determine the Mn(III) reduction

activity of mutant strains that lack specific electron transport and protein secretion genes involved in Fe(III) and Mn(IV) reduction (Chapter 3), iii) determine if Mn(IV) reduction to Mn(II) proceeds step-wise through two one-electron transfer reactions with Mn(III) as transient intermediate (Chapter 4), and iv) enrich for microbial consortia that couple NH_4^+ oxidation to Mn(III) reduction (Chapter 5).

A suite of Mn(III) reduction-deficient mutant strains were isolated, including Mn(III) reduction-deficient mutant strain Mn3-1 that also displayed the ability to reduce soluble organic-Fe(III), but not solid Fe(III) oxides, demonstrating for the first time that the reduction of soluble organic-Fe(III) and solid Fe(III) oxides proceed through electron transport pathways with at least one distinct component (Chapter 2). This work also shows that the electron transport pathway for Mn(III) reduction in *S. oneidensis* shares many of the electron transport components of Fe(III) and Mn(IV) reduction pathways (Chapter 3) and that Mn(IV) reduction to Mn(II) proceeds step-wise through two one-electron transfer reactions with Mn(III) as a transient intermediate (Chapter 4). Finally, a microbial consortia was identified that coupled the oxidation of NH_4^+ to the reduction of Mn(III). The consortium was dominated by an electrogenic *Ochrobactrum* sp. and a Mn(III)-reducing *Shewanella* sp. that potentially coexist in a syntrophic association. The isolated *Shewanella* strain is able to oxidize acetate with Mn(III) as electron acceptor, an activity never observed before in a metal-reducing member of the *Shewanella* genus.

CHAPTER 1

INTRODUCTION

Biochemical cycling of manganese

Manganese (Mn) deposits are ubiquitous throughout the oceans [1-4], soils and sediments [5]. Mn deposits originate from geological processes (hydrothermal vents and volcanic activity) and microbial activity. Mn is a strong adsorbant and oxidant of metals, radionuclides and organic compounds. The biogeochemical cycling of Mn thus plays a central role in the fluxes of organic carbon, nutrients and organic and inorganic contaminants [3, 6-8]. Microorganisms may accelerate Mn redox transformations by orders of magnitude [1] and, consequently, greatly influences the biogeochemical fluxes of other metals, carbon, and many trace elements in the environment [9-13].

Heterotrophic Mn(IV) respiration, for example, is the dominant process for organic carbon remineralization in Mn-rich sediments [2, 14-17]. Microbial Mn reduction is also postulated to be an ancient respiratory process on early Earth. Understanding the flux of Mn may therefore provide insight into the biogeochemical history of Earth. For example, fossil records of bacteria, the co-occurrence of Mn with organic carbon and ubiquitous sedimentary Mn deposits throughout geological history [18] provide geological evidence for the existence of microbial Mn reduction on early Earth. Phylogenetic analyses showing deeply rooted 16S rDNA sequences of dissimilatory metal reducing bacteria (DMRB) and the presence of *Archaea* with the ability to reduce metals [10, 19] support the hypothesis that Mn transformations during early Earth were microbially-driven. Mn oxidation has, presumably, an anoxic origin as well [20], although this claim has been disputed [21]. Soluble Mn(III) may have an ancestral origin and may have participated in oxygen evolution in the planet, perhaps even prior to photosynthesis.

Traditional models describe the Mn distribution in the environment as a consequence of biological and abiotic redox transformations between solid Mn(III, IV) oxides and soluble Mn(II), which can precipitate with CO_3^{2-} or PO_4^{3-} [8, 22] depending on the concentration of these ions in solution. Mn(II) is formed in anoxic environments by bacterially mediated Mn(III, IV) reduction coupled to organic carbon oxidation in anaerobic zones [6, 18, 23] and diffuses to surface waters, where it becomes reoxidized by O_2 and precipitates back to the sediments [22], where it is typically found in the form of Mn-enriched granules [1]. Mn oxides are readily reduced by disulfides, FeS(s) and Fe(II) found in anoxic sediments. The distribution and redox transformations of Mn(III) oxides are not well characterized [24]. The presence of soluble Mn(III), on the other hand, has been assumed to be negligible, because Mn(III) is highly unstable and rapidly disproportionates to Mn(II) and Mn(IV) in the absence of stabilizing ligands [25, 26]. Recent voltammetric-based detection techniques, however, have found that ligand-bound Mn(III) often dominates the soluble Mn pool in aquatic environments, in some cases representing up to 100% of the total pool [25, 27, 28]. Chelated Mn(III) may originate from debris of Mn-containing enzymes such as the water-oxidizing complex of photosystem II, catalases and superoxide dismutases, as a transient intermediate during oxidation and reduction of Mn [29], or by complexation with organic compounds at hydrothermal vents [30]. Microbial Mn(III) transformations may therefore represent a major, yet overlooked redox process in anaerobic marine and freshwater environments. Previous studies have focused on Mn-reducing microbial populations in the environment [2, 31]. In these studies, carbon source utilization was measured and correlated to the presence of populations in Mn-rich sediments, but direct empirical evidence was not used

to link carbon oxidation to metal reduction by these microorganisms. Metabolic activity in the environment can only be studied by linking substrate consumption with microorganism identity when the substrate is incorporated into cell components such as DNA or fatty acids [32]. Metal reduction, however, is a dissimilatory process, and this strategy cannot be employed. Alternatively, populations can be followed by determining the presence or expression of functional genes, but metal reduction genes display high sequence divergence, which limits their utility as molecular markers for all DMRB [33]. Metal-reducing marker genes are available for specific subgroups and include those encoding citrate synthase (GltA) of the metal-reducing *Geobacteraceae* family [34] and the beta-barrel protein MtrB of metal-reducing *Gammaproteobacteria* which contains a diagnostic N-terminal CXXC motif [35]. Tracking the presence and activity of all DMRB in the environment, however, is still an unresolved methodological challenge.

Microbial manganese reduction

Microbial metal reduction

DMRB reduce many metal oxides in sparingly soluble amorphous or crystalline form, such as Mn and Fe [26]. Mn(IV) and Mn(III) reduction by heterotrophic bacteria are thermodynamically favorable and highly endergonic at pH 7 (0.43 eV and 0.51 eV respectively). Correspondingly with the reduction potential of Mn and Fe oxides, Mn and Fe reducing bacteria can be found above sulfate reduction and below nitrate reduction zones in redox stratified environments[36] . Insoluble metals oxides are reduced extracellularly, and many DMRB can also respire anaerobically on other soluble compounds present outside the cell such as DMSO, flavins, and humic substances [37].

Extracellular respiration is a subject of interest for applications such as microbial fuel cells [17, 38] and bioremediation of contaminants such as dyes [39, 40], radionuclides [41] and chlorinated compounds [42]. Despite the environmental significance of DMRB, however, the molecular mechanism of extracellular respiration by bacteria remains poorly understood.

The two mechanisms of ATP generation in living organisms include substrate level phosphorylation and electron transport chain-linked (oxidative) phosphorylation. The design of the aerobic electron transport chain is similar in eukaryotes and prokaryotes. Respiration of oxygen involves the transfer of electrons along redox-active electron carriers of increasingly positive reduction potential, located on the inner membrane. The transfer of electrons is linked to proton translocation across the membrane, generating an electrochemical potential that results in the formation of ATP by a membrane-bound ATPase when the proton gradient is dissipated by controlled release of the protons back into the cell [43]. Both the oxidation of a substrate and the final disposal of the electrons occur on the cytoplasmic side in the cell (or lumen of mitochondria). The free energy of electron transfer is determined by the redox potential of the electron donor-acceptor pairs. Reduction potentials for the major redox couples involved in microbial redox processes are displayed in Table 1.1. During extracellular respiration, however, electrons that originate from the oxidation of a substrate on the cytoplasmic side are transferred to acceptors located outside of the cell. In this pathway, electrons are conducted across a distance of at least 140Å that includes both inner and outer bacterial membranes and the periplasmic space [44]. Thus, for extracellular respiration by bacteria, electron transport systems different than the inner membrane-

localized electron transport chains [43] must be employed [45, 46]. In addition, many metal oxides are insoluble at circumneutral pH, adding a kinetic barrier for reduction. *S. oneidensis* has evolved a specific respiratory strategy, known as the metal-reducing (Mtr) pathway, composed of a network of *c*-type cytochromes located on the inner membrane, periplasm, and outer membrane that facilitates extracellular electron transport to both solid and soluble extracellular electron acceptors [47-49] (Figure 1.2). Cytochromes are heme proteins with (proto)porphyrin groups axially ligated by coordination bonds and, in the case of *c*-type cytochromes, covalent bonds [50]. Heme proteins are redox-active molecules that display reduction potential ranges typically spanning from -550 mV to $+450$ mV versus SHE. Heme protein E_m values are a function of the type of global protein fold, the type of porphyrin and the identity of the axial ligand, all of which can vary their redox window up to 800 mV [51] .

In the Mtr pathway, primary dehydrogenases transfer electrons to menaquinone, a modified form of quinones used during anaerobic respiration [43]. Menaquinol reduces CymA [51], a small (21 kDa) tetraheme cytochrome [52] associated with the inner-membrane with the haem-containing globular domain located in the periplasmic space [53]. CymA acts as a respiratory hub [54, 55] that transfers electrons directly to a number of *c*-type cytochromes in the periplasm that terminate in the periplasmic space or at the extracellular side with reduction of fumarate, NO_3^- , NO_2^- , DMSO, Fe(III), and Mn(IV) as terminal acceptors [56, 57]. During metal reduction, CymA transfers electrons to MtrA, a decaheme cytochrome located on the inner side of the OM. Expression of MtrA is required for proper MtrB positioning in the outer membrane [54]. MtrB is an OM transmembrane protein [55], with 28 to 33 β -barrel strands essential for metal reduction

[33]. Homology models show that MtrB forms a cylindrical tube that functions as an anchor point and protective sheath that allows MtrA and MtrC to interact with each other at the outer membrane [58]. MtrB, in addition, is required for proper localization of MtrC and OmcA [59, 60]. MtrC and OmcA are two decaheme *c*-type cytochromes located on the extracellular side, and the final components of the Mtr electron transport chain [59-61]. Purified forms of MtrC and OmcA reduce soluble and solid Fe(III) [45, 62, 63] and may serve as terminal metal reductases by direct contact of external metal oxides [64, 65], or involved indirectly in electron-acceptor reduction via endogenous or exogenous redox-active electron shuttles. Shuttles such as humic acids and flavins [65-68] participate in a redox loop, recycling between the terminal reductase and the terminal electron acceptor [61, 69, 70]. Purified MtrB, MtrC, and MtrA form a lipid-embedded complex in proteoliposomes that transfers electrons across the lipid membrane to soluble Fe(III) substrates [71-74], thus suggesting that MtrA, MtrB, and MtrC operate in the same manner in vivo, forming a conduit that transfers electrons from the periplasm across the outer membrane to the extracellular side where they transfer electrons to the final electron acceptor [61, 67, 75, 76]. Experimental evidence for the Mtr pathway in metal reduction is focused mainly on Fe(III) reduction. The involvement of CymA, OmcA, and MtrCAB in Mn(III) reduction is not known and is the subject of chapter 3 of the thesis work.

Table 1.1. Selected reduction potential of relevant electron donors, electron carriers and electron acceptors of bacterial respiration. Electron donors focused on in this thesis are in bolded italics, and electron acceptors in bold. Adapted from [43, 77].

Electron carrier/acceptor (reduced)	Reduction potential	Reduction product (oxidized)
CO ₂ ⁻	-0.43	<i>Formate</i>
H	-0.421	<i>H₂</i>
Ferredoxin ox	-0.39	
Cystine	-0.34	Cysteine
NAD ⁺	-0.32	NADH ⁺ + H ⁺
CO ₂ ⁻	-0.29	<i>Acetate</i>
CO ₂ ⁻	-0.24	Methane
Fe(OH) ₃	-0.236	Fe(II)
Pyruvate	-0.185	<i>lactate</i>
Oxaloacetate	-0.166	Malate
Menaquinone	-0.075	Menaquinol
Fumarate	0.031	Succinate
FAD	0.031	FADH ₂
Ubiquinone	0.045	ubiquinol
Cytochrome <i>b</i>	0.07	
Cytochrome <i>c</i>	0.254	
NO ₂ ⁻	0.385	NO
Cytochrome <i>a</i>	0.385	
SO ₄ ²⁻	0.52	HSO ₃ ⁻
Mn(IV)	0.798	Mn(II)
O ₂	0.816	H ₂ O
NO	1.18	N ₂ O
N ₂ O	1.36	N ₂
Mn(III)	1.51	Mn(II)

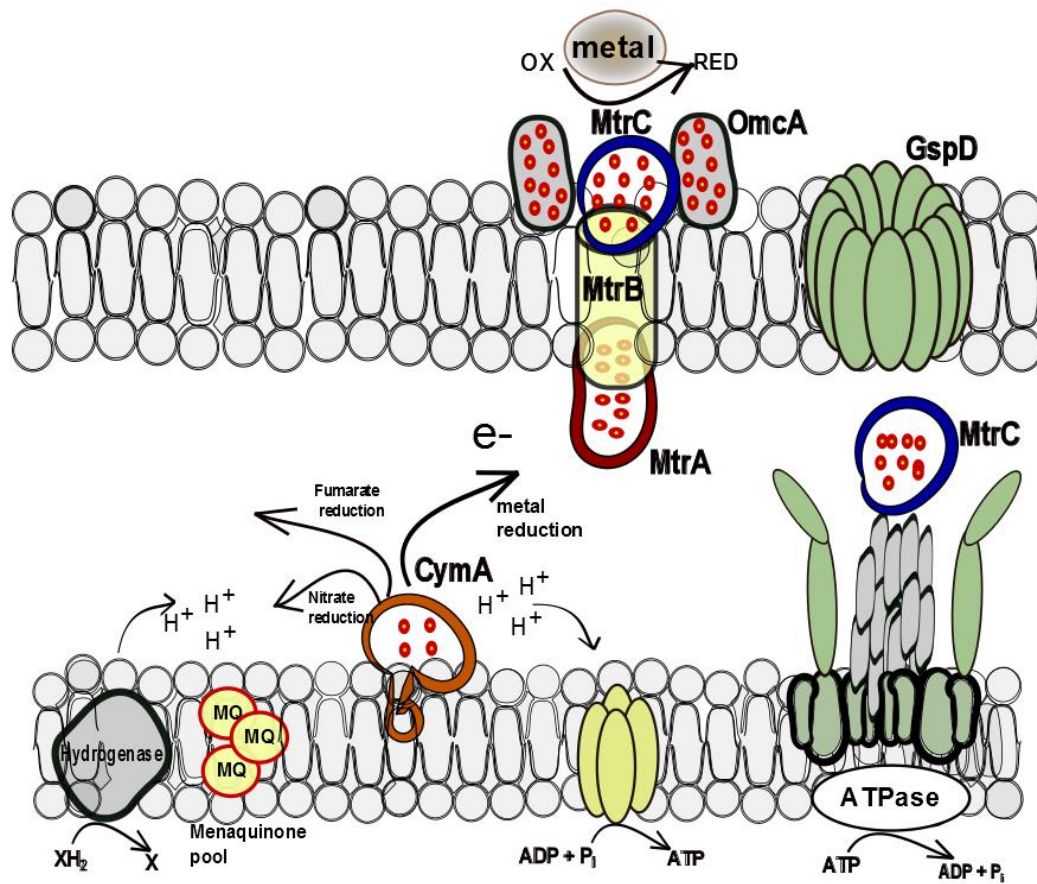


Figure 1.2. Schematic representation of the Mtr electron transport pathway in *S. oneidensis* MR-1. Adapted from [55, 61, 78].

Role of protein secretion pathways in microbial metal reduction

The reduction of extracellular electron acceptors requires that electrons be transported across the OM. Protein secretion systems are involved in the transport and correct positioning of OM or extracellular proteins [79]. At least three secretion systems, Type I, Type II, and Type V, participate in extracellular respiration in *S. oneidensis*. TolC is a type I, channel-forming trimeric secretory protein that functions as a general efflux pump to secrete antimicrobial agents and metals in *E. coli*. [80, 81]. In *S. oneidensis*, TolC is required for AQDS reduction because it is responsible for secretion of the toxic end-product AH₂DS [82]. The role of TolC in metal reduction, however, has not been studied. The Type II secretion system (T2SS) is a two-step ATP-dependent process that translocates exoproteins to the cell surface or extracellular milieu by the pumping action of a pseudopilus [62] and it is postulated to translocate MtrC and OmcA to the outside face of the OM [65] (Figure 1.2). *S. putrefaciens* mutants lacking the T2SS gene *gspE*, which encodes a traffic ATPase, are unable to respire Fe(III) and Mn(IV) oxides but retain the ability to reduce electron acceptors in the periplasm, such as NO₃⁻ [83-85]. In metal-reducing *S. oneidensis* the OM secretin GspD is essential for Fe(III) reduction, but its role in Mn(III) reduction has not been studied. In *S. oneidensis*, SO_3800 encodes a Type V secretion autotransporter, a subtilisin-like serine protease that is required for adhesion to solid substrates including Fe(III) oxides. *S. oneidensis* in-frame deletion mutants lacking SO_3800 (Δ SO3800) are unable to attach to Fe(III) oxides, presumably due to an impaired production of exopolysaccharides. The role of SO_3800 in Mn(IV) or Mn(III) respiration is not known .

Mn(III) as a transient intermediate during Mn(IV) reduction

Molecular orbital analyses predict that redox transformations between Mn(VI) and Mn(II) proceed via two consecutive one-electron transfer reactions, and not via one single, two-electron transfer reaction. In the case of Mn(IV) reduction, the first electron is added to one of the two empty e_g orbitals which is reduced to Mn(III) as it undergoes orbital rearrangement and a second electron reduces Mn(III) to Mn(II) [86]. The molecular orbital analyses are supported by experimental studies that have shown that Mn(III) is a transient intermediate during extracellular Mn(II) oxidation to Mn(IV) by multicopper oxidases located on the extracellular side of the exosporium of spores of the marine *Bacillus* sp strain SG-1 [29]. In this strain, Mn(II) and Mn(III) oxidation reactions, while energetically distinct, are catalyzed by the same multicopper oxidase enzymatic complex [87]. An analogous electron transfer mechanism may be in place in *S. oneidensis* during Mn(IV) reduction to Mn(II). In addition, iron oxide involves a non-reductive solubilization step prior to reduction, which presumably increases surface area and reduces crystallinity, resulting in more readily reducible oxides. Correspondingly, a reductive solubilization step may be involved in Mn(IV) oxide reduction. No Mn(IV) ligand nor soluble Mn(IV), however, has been detected in the environment. Therefore, solubilization would require an initial reductive dissolution step of Mn(IV) to Mn(III) suggesting that Mn(III) is the energy-generating species, analogous to soluble Fe(III) citrate.

***Shewanella* in the environment**

Shewanella are gram-negative *Gammaproteobacteria*, ubiquitously distributed in a variety of aquatic environments, including freshwater and marine sediments [88, 89] and are also present in extreme environments such as hydrothermal vents [90], the deep sea [91] and even human clinical samples [17, 92]. The *Shewanella* genus is currently composed of 61 species with accepted nomenclature [93]. New species designations are based primarily on DNA:DNA hybridization and 16S rRNA sequences, and 22 strains representing 8 species have been fully sequenced. *Shewanella* have been isolated from around the globe and all of the isolated species have been classified as facultative anaerobes [17]. Members of the *Shewanella* genus are strict respirers that can grow both aerobically and anaerobically [17, 92], although ATP may also be produced by substrate level phosphorylation via acetate kinase [94]. *Shewanella* are known to be competitive in redox stratified environments. In anaerobic ecosystems, specific bacterial populations are responsible for organic matter degradation: Extracellular hydrolytic degradation of polymers is followed by fermentation of the monomers or oligomers to mainly volatile fatty acids (VFAs) and H₂. Oxidation of VFAs and H₂ is coupled to anaerobic respiration to produce CO₂ and alternative byproducts [59, 95]. The limited ability of *Shewanella* to uptake and oxidize carbon compounds (mainly C1-3 carbon compounds) [96], coupled to respiration of a wide range of electron acceptors limits their ecological niche as consumers of organic matter breakdown products [97, 98]. *Shewanella* can also use amino acids and DNA as sole carbon sources [99], and oligopeptides as sole nitrogen sources [88]. Some *Shewanella* species can also degrade halogenated compounds and

nitroaromatic compounds. *S. oneidensis* for example, is predicted to use 33 compounds as sole carbon and energy source under aerobic conditions. *Shewanella* are known for their metal-reducing ability, and are also able to respire more than 20 compounds [88], including organics such as azo dyes [100], and has been used extensively as a research model for extracellular respiration. This respiratory versatility and the presence of a large number of chemotaxis and chemoreceptors genes compared to most other sequenced *Gammaproteobacteria* [88] are further evidence that *Shewanella* are adapted to redox-stratified environments. The metabolic versatility of *Shewanella* may possibly be due to high rates of horizontal gene transfer[101]. An analysis of various *Shewanella* species showed that only 40% of their core genome is shared with each other, suggesting a high potential to uncover new genes [102]. The majority of the biochemical characterizations of *Shewanella*, however, have been performed on *S. oneidensis* MR-1, *Shewanella* sp. ANA3 and *S. putrefaciens* CN32. The biochemical potential of the *Shewanella* genus is therefore largely untapped.

The only account of Mn(III) reduction to date belongs to reports of Mn(III)-pyrophosphate reduction by *S. oneidensis* MR-1 [77] which was isolated from an enrichment culture with solid Mn(IV) oxides as electron acceptor [103]. *S. oneidensis* MR-1 is able to use lactate, formate and H₂ as electron donor. Lactate is converted to pyruvate, and hydrogenases transform pyruvate to acetate and formate, which is subsequently converted to H₂ and CO₂ [104, 105]. *S. oneidensis* MR-1 cannot use acetate as electron donor during anaerobic respiration. *S. loihica* and *S. denitrificans* are the only *Shewanella* species observed to oxidize acetate anaerobically to NO₃⁻ reduction [106]. While microbial Mn(III) transformations may represent a major, yet overlooked redox

process in marine and freshwater systems, little is known about the identity of Mn(III)-reducing populations in the environment.

Biogeochemical cycling of nitrogen

Microorganisms catalyze the cycling of nitrogen in the environment, which is tightly coupled to cycles of other biologically important elements including carbon and phosphorus, which directly impact primary productivity and greenhouse gas dynamics [107, 108]. The known pathways of the nitrogen cycle include 1) anaerobic reduction and transformation of N_2 gas to ammonia (nitrogen fixation), 2) conversion of inorganic NH_3 into organic forms (assimilation), 3) oxidation of NH_4^+ as electron donor to NO_2^- with O_2 as electron acceptor, 4) oxidation of NO_2^- to NO_3^- (nitrification) with O_2 as electron acceptor, 5) anaerobic oxidation of NH_4^+ to N_2 with NO_2^- as electron acceptor (Anammox), 6) anaerobic reduction of NO_2^- and NO_3^- to N_2 (denitrification), and 7) reduction of NO_2^- to ammonia (ammonification) [43, 109]. The diversity in nitrogen metabolic pathways is hypothesized to have originated during anaerobic conditions on early Earth: Combustion processes generate NO_3^- and NO_2^- and NH_4^+ is produced from N_2 at hydrothermal vents [110, 111] giving rise to the anaerobic pathways (1, 2, 5, and 6 above). Later, oxygenation of the atmosphere by cyanobacteria provided the oxidant that resulted in evolution of new sets of biochemical reactions, including aerobic microbial NH_4^+ oxidation to NO_3^- and NO_2^- . Many of the anaerobic pathways are still prevalent in the modern nitrogen cycle in the anaerobic niches of the planet, such as the Anammox reaction which may be the dominant pathway to N_2 in many anaerobic environments

[111-113] The evolutionary history of these biochemical pathways, however, is not clear. For example, Anammox processes depend on production of NO_2^- from NO_3^- and although the process is anaerobic, it may have arisen after the rise of O_2 in the atmosphere, which is necessary for NO_3^- production. However, the recent finding of methane oxidation coupled to NO_2^- reduction in anaerobic environments is postulated to proceed via an aerobic biochemical pathway, with O_2 formed from the reaction of 2NO and N_2 as a byproduct [114]. An analogous mechanism may be operating for NH_4^+ oxidation but has yet to be discovered. This cryptic aerobic pathway for methane oxidation suggests that aerobic respiratory processes may have evolved under anaerobic conditions before the great oxidation event (GOE).

Interactions between N and Mn cycles

Thermodynamic calculations of predicted anaerobic respiratory pathways indicate that some metal-nitrogen redox couples provide sufficient free energy for microbial growth. Among these, energy-generating pathways involving the transition metal Mn and nitrogen have yet to be fully explored, in particular the oxidation of NH_4^+ coupled to the reduction of Mn as electron acceptor [115]. A summary of the thermodynamically feasible reactions are included in Table 1.2 and a graphic summary is shown in Figure 1.2. Microbially-catalyzed NH_4^+ oxidation coupled to Mn(IV) or Mn(III) reduction leading to production of N_2 or NO_2^- and Mn(II) are thermodynamically favorable reactions that yield more than the 31.8 kJ/mol required for ATP production [43]. Porewater profiles of Mn, NH_4^+ , and NO_2^- suggest that NH_4^+ oxidation coupled to Mn

oxide reduction exists in nature [115, 116]. Despite this geochemical data, the biotic nature of Mn-driven NH_4^+ oxidation reactions is still under debate. Furthermore, a microorganism displaying this metabolism has not yet been cultivated. The proposed novel Mn-driven NH_4^+ oxidation pathway is analogous to those proposed for anaerobic CH_4 oxidation coupled to Fe(III) and Mn(IV) reduction .

Table 1.2 Thermodynamic calculations of theoretical redox reactions between nitrogen species .

$\text{NH}_4^+ + \frac{3}{2}\text{MnO}_2 + 2\text{H}^+ \xrightarrow{\text{yields}} \frac{3}{2}\text{Mn}^{2+} + \frac{1}{2}\text{N}_2 + 3\text{H}_2\text{O}$	-209.45	} NH_4^+ oxidation coupled to Mn reduction
$\text{NH}_4^+ + 3\text{MnOOH} + 5\text{H}^+ \xrightarrow{\text{yields}} 3\text{Mn}^{2+} + \frac{1}{2}\text{N}_2 + 6\text{H}_2\text{O}$	-156.66	
$\text{NH}_4^+ + 3\text{MnO}_2 \xrightarrow{\text{yields}} 3\text{MnOOH} + \frac{1}{2}\text{N}_2 + \text{H}^+$	-61.1	
$\text{NH}_4^+ + 6\text{MnO}_2 + 2\text{H}_2\text{O} \xrightarrow{\text{yields}} 6\text{MnOOH} + \text{NO}_2^- + 2\text{H}^+$	-262.2	
$\text{NH}_4^+ + 6\text{MnOOH} + 10\text{H}^+ \xrightarrow{\text{yields}} 6\text{Mn}^{2+} + \text{NO}_2^- + 10\text{H}_2\text{O}$	-166.67	
	44.47	

While nitrification is often described as an O_2 -dependent pathway, it may also proceed via hydroxylamine in an NO_2^- -dependent pathway under anaerobic conditions [107, 117, 118]. *Nitrosomonas europaea*, for example, carries out NO_2^- -dependent ammonia oxidation under anaerobic conditions (even in the presence of acetylene, a potent inhibitor of ammonia monooxygenase). A second pathway, commonly known as

the Anammox pathway, involves the oxidation of NH_4^+ coupled to NO_2^- reduction with the production of hydrazine which is transformed to N_2 [119]. A third possibility involves a pathway analogous to CH_4 oxidation coupled to NO_2^- reduction in which O_2 is produced by an as yet unknown enzyme which dismutates 2NO to N_2 and O_2 , and subsequent activation of CH_4 via methane monooxygenase for subsequent oxidation reactions following the well-known aerobic methane oxidation pathway [114]. An analogous mechanism may be operating for NH_4^+ oxidation coupled to Mn reduction. In fact, Mn interacts with dioxygen (and partially reduced derivatives) in many biological systems, such as the O_2 -evolving complex (OEC) in photosystems, catalases, dismutases, and nucleotide reductases [120, 121]. These three pathways for NH_4^+ oxidation may, hypothetically, also operate with MnO_2 in the place of NO_2^- . NH_4^+ oxidation coupled to Mn(IV) reduction has been proposed previously: coincident peaks of NO_2^- (the product of NH_4^+ oxidation) and Mn(II) (the product of Mn(III) or Mn(IV) reduction) near the surface/water interface (SWI) and the absence of NO_3^- in sediment pore waters at several sites (including Skidaway Institute of Oceanography [122] and Saguenay Fjord in the St. Lawrence estuary) may be explained by microbially-mediated anaerobic NH_4^+ oxidation coupled to Mn(IV) reduction.

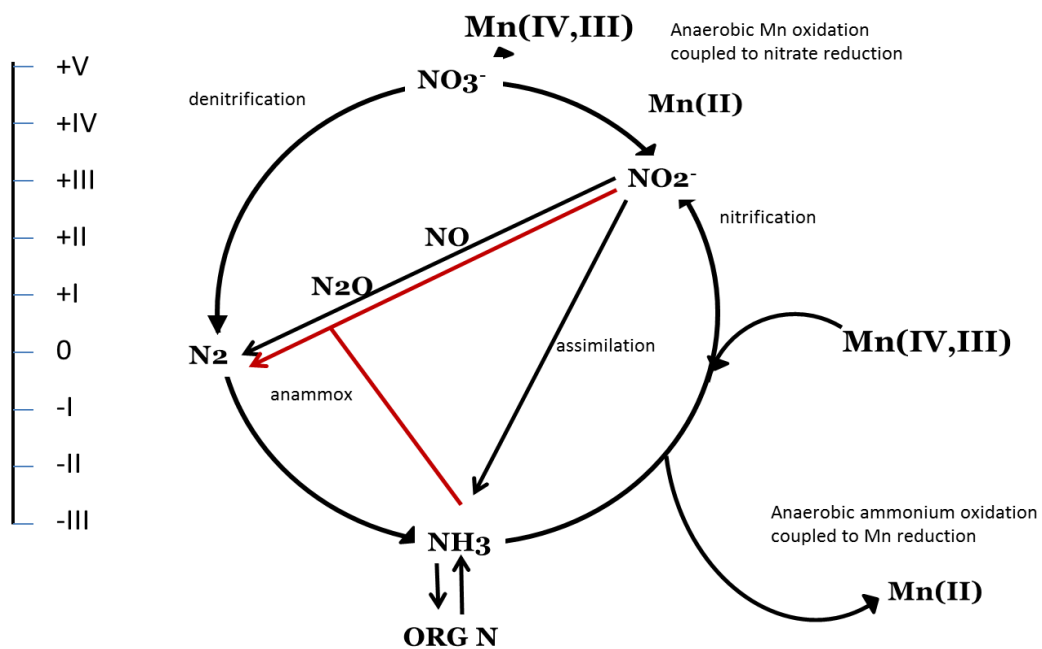


Figure 1.2 Proposed interactions of Mn with the N cycle.

Research objectives

The overall aim of the thesis was to determine the molecular mechanism of microbial Mn respiration and identify microbial consortia that couple the biogeochemical cycling of Mn and nitrogen. To meet this overall aim, the specific objectives of the thesis were to i) generate Mn(III) reduction-deficient mutant strains and identify genes involved in Mn(III) reduction (Chapter 2), ii) determine the Mn(III) reduction activity of mutant strains that lack specific electron transport and protein secretion genes involved in Fe(III) and Mn(IV) reduction (Chapter 3), iii) determine if Mn(IV) reduction to Mn(II) proceeds step-wise through two one-electron transfer reactions with Mn(III) as transient intermediate (Chapter 4), and iv) enrich for microbial consortia that couple NH_4^+ oxidation to Mn(III) reduction (Chapter 5).

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CHAPTER 2

DESIGN OF A RAPID SCREENING TECHNIQUE FOR ISOLATION OF MN(III) REDUCTION-DEFICIENT MUTANTS

Microbial Mn transformations greatly impact the biogeochemical cycles of metals, carbon, and other trace elements. Although the presence of soluble Mn(III), has been traditionally assumed negligible, recent voltammetric-based detection techniques, however, have found that ligand-bound Mn(III) often dominates the soluble Mn pool in aquatic environments, and in some cases represents up to 100% of the total soluble Mn pool. Microbial Mn(III) transformations may therefore represent a major, yet overlooked redox process in aquatic environments. Although previous studies have reported that *S. oneidensis* can reduce soluble Mn(III), and despite the environmental significance of Mn(III) reduction, the molecular mechanism of Mn(III) respiration by bacteria has not been previously examined. The present study describes the development of a rapid screening technique to identify Mn(III) reduction-deficient mutants of *S. oneidensis*. Several Mn(III) reduction mutants were identified and Mn3-1-5 were confirmed in liquid culture. Mn3-1 was unable to reduce soluble Mn(III) or solid Fe(III) oxide, but retained the ability to reduce soluble Fe(III). Mn3-1 is the first metal reduction-deficient mutant in any metal-reducing bacterial strain that genetically separates the ability to reduce soluble and solid metals. Genetic analysis of Mn3-1 revealed several single nucleotide mutations. In frame deletion of candidates RTX toxin and formate dehydrogenase did not result in a Mn(III) reduction-deficient phenotype. A complementing genomic fragment, however, was identified via genetic complementation. The identity of the complementing gene needs to be obtained.

Introduction

Microbial Mn transformations greatly impact the biogeochemical cycles of metals, carbon, and other trace elements [1]. For example, heterotrophic Mn(IV) respiration can be a dominant process for organic carbon remineralization in Mn-rich sediments [2, 3]. Microbial Mn redox transformations affect Mn chemical speciation, and subsequently, Mn interaction with many organic compounds [4-7], many of which are emerging contaminants [8]. In addition, changes in Mn speciation and solubility properties influence formation, dissolution, and adsorption of other minerals [9-14]. Traditional models describe Mn distribution in the environment as a consequence of biotic and abiotic redox transformations between solid Mn(III, IV) oxides and Mn(II), which remains as soluble species, or precipitates with CO_3^{2-} or PO_4^{3-} [1, 15, 16] depending on the concentration of these ions in solution. Mn(II) is formed in anaerobic environments by bacterially mediated Mn(III, IV) reduction coupled to organic carbon oxidation in anaerobic zones [17, 18] and diffuses to surface waters, is reoxidized by O_2 and precipitates back to the sediments [16] where freshly oxidized Mn is typically found in the amorphous oxide form [5]. The distribution and redox transformations of Mn(III) oxides are not well characterized [19]. The presence of soluble Mn(III), on the other hand, has been assumed negligible, because soluble Mn(III) is highly unstable and rapidly disproportionates to Mn(II) and Mn(IV) in the absence of stabilizing ligands [20, 21]. Recent voltammetric-based detection techniques, however, have found that ligand-bound Mn(III) often dominates the soluble Mn pool in aquatic environments, and in some cases represents up to 100% of the total soluble Mn pool [20, 22, 23]. Microbial Mn(III) transformations may therefore represent a major, yet overlooked redox process in aquatic

environments. The ability to enzymatically reduce metal oxides in sparingly soluble amorphous or crystalline form such as Mn(IV) is restricted to a relatively small number of dissimilatory metal-reducing bacteria (DMRB), including members of the *Shewanella* genus. *S. oneidensis* MR-1 reduces a variety of transition metals and radionuclides, including Fe(III), Mn(IV), U(VI), and Tc(VII). *S. oneidensis* also respire soluble non-metal compounds such as DMSO, flavins, and humic substances. Although previous studies have reported that *S. oneidensis* can reduce soluble Mn(III), and despite the environmental significance of Mn(III) reduction, the molecular mechanism of Mn(III) respiration by bacteria has not been previously examined.

Fe(III)- and Mn(IV)-reducing bacteria are presented with a unique physiological challenge at circumneutral pH: they are required to reduce electron acceptors found largely as amorphous or crystalline (oxy)hydroxide particles presumably unable to contact inner membrane (IM)-localized electron transport systems, where hydrogenases, menaquinone and ATPase are localized. To overcome this problem, metal-reducing bacteria employ novel electron transport strategies not found in other gram-negative bacteria that reduce soluble electron acceptors including 1) direct enzymatic reduction of solid metal oxides via metal reductases localized on the outer membrane (OM) or on electro-active appendages [24-27], 2) a two-step, solubilization and reduction pathway in which solid metal oxides are first dissolved by organic complexing ligands, followed by uptake and reduction of the soluble organic-metal complexes by periplasmic metal reductases [28-30], and 3) a two-step, electron shuttling pathway in which exogenous or endogenous electron shuttling compounds are first enzymatically reduced and then

chemically oxidized by the solid metal oxides in a second (abiotic) electron transfer reaction [31-33].

Molecular orbital analyses suggest that the final electron transfer to Mn(IV) reduction proceeds via Mn(III) as a transient intermediate [34]. Understanding the biochemical differences of Mn(IV) and Mn(III) reduction is, therefore, essential for predicting Mn transformations in the environment. Extracellular metal reduction is a subject of great interest for applications such as microbial fuel cells, and bioremediation of contaminants such as antibiotics, dyes, radionuclides, and chlorinated compounds [8, 15, 35]. The present study describes the development of a rapid screening technique to identify Mn(III) reduction-deficient mutants of *S. oneidensis*. Genetic analysis of the Mn(III) reduction-deficient mutants will provide fundamental information on the electron transport chain components required for microbial Mn(III) reduction.

Materials and methods

Bacterial strains and growth conditions

S. oneidensis MR-1 was originally isolated from Lake Oneida, NY [36] and was employed in the present study as the parent strain for chemical mutagenesis. Bacterial strains and plasmids used in the present study are listed in Table 2.1. For genetic manipulations, *E. coli* and *S. oneidensis* strains were cultured in Luria Bertani (LB) medium at 37 °C and 30 °C, respectively. For anaerobic electron acceptor reduction experiments, *S. oneidensis* strains were grown in M1 medium supplemented with lactate (18 mM) as electron donor. Amorphous Mn(IV) oxides were synthesized by reaction of MnCl₂ with KMnO₄ followed by neutralization with NaOH [37, 38]. Mn(III) was prepared by solubilization of Mn(III)-acetate, followed by complexation with excess sodium pyrophosphate [39]. Poorly crystalline Fe(III) oxides and soluble Fe(III) citrate were synthesized by neutralizing a solution of FeCl₃ and Fe(III)-citrate, respectively, with NaOH to pH 7 [40].

Isolation of Mn(III) reduction-deficient mutant strains of *S. oneidensis*

Ethyl methane sulfonate (EMS) was used as a chemical mutagen following previously described procedures. Liquid cultures of wild-type *S. oneidensis* were grown in M1 medium to late exponential phase (2×10^9 cells mL⁻¹), harvested by centrifugation (4 °C), washed twice, and resuspended in fresh M1 medium. EMS was added (19 mg/mL) and the cell suspension was incubated 45 min to achieve 90% kill. The surviving EMS-treated cells were plated on SM agar and the colonies were subsequently subjected to a

mutant screening technique to detect loss of Mn(III) reduction activity. Approximately 5,000 EMS-treated colonies were transferred to SM medium supplemented with 18 mM lactate as electron donor and 10 mM Mn(III)-pyrophosphate as electron acceptor. After 48 hours of incubation in a Coy anaerobic chamber (atmosphere of 5% CO₂, 10% H₂, 85% N₂), the wild-type colonies produced a distinctive clearing zone, an indication that pink-colored Mn(III)-pyrophosphate had been reduced to colorless Mn(II) end-products, while five putative Mn(III) reduction-deficient mutants were identified by their inability to produce a clearing zone. One of the five putative Mn(III) reduction-deficient mutants (designated strain Mn3-1) was selected and subsequently tested for Mn(IV) reduction, Mn(III) reduction, and DIC production activities in anaerobic liquid cultures supplemented with 18 mM lactate and either 7 mM Mn(IV) oxide or 7 mM Mn(III)-pyrophosphate.

Identification of single nucleotide mutations in mutant strain Mn3-1

Mn3-1 genome sequencing was carried out at the University of Illinois-Chicago by Dr. Stefan Green [41]. Genomic DNA was prepared for shotgun sequencing using the Nextera DNA sample preparation kit (Epicenter, Madison, WI), according to manufacturer's instructions. Subsequently, size selection was performed to capture 400- to 800-bp fragments using a Pippin Prep automated electrophoresis instrument (Sage Scientific, Beverly, MA). The generated library was quantified using quantitative PCR with the KAPA library quantification kit. The library was sequenced as a portion of an Illumina HiSeq2000 lane, using 100-base paired-end sequencing. Approximately 61 M reads in pairs were recovered.

Raw sequence data was processed within the software package CLC genomics workbench (V6). Sequences were trimmed using high stringency (Q20), and sequences with any poor quality bases were removed from the dataset. After trimming, approximately 39M reads remained. De novo assembly was performed within CLC genomics using default parameters. 97.5% of all reads were incorporated into the de novo assembly, generating 259 contigs of total length of 4,886,023 bases. The maximum contig was 255,408 bases in length, and the N50 of the assembly was 45,082 bases. The estimated insert size based on the assembly range from 220-670, with a maximum near 350 bases.

The Mn3-1 genome was compared to the wild type genome sequence (NCBI database) using Bowtie, a short-read mapping software [42] with the assistance of Dr. Konstantinidis lab member Luis Rodriguez Rojas. Single nucleotide changes were retrieved as locus site numbers. Each mutation and its corresponding gene were confirmed manually using the NCBI database.

Table 2.1. Strains and plasmids used in this study

Strain	Features	Source
<i>S. oneidensis</i>		
MR-1	Wild-type strain	ATCC
$\Delta fdnG$	In-frame <i>fdnG</i> deletion mutant	This study
ΔRTX	In-frame <i>rtx</i> deletion mutant	This study
<i>E. coli</i>		
$\beta 2155 \lambda$ pir	<i>thrB1004 pro thi strA hsdS lacZ_M15(F9 lacZΔM15 lacIq traD36 proA1 proB1) $\Delta dapA::erm pir::RP4$ Km^R</i>	[43]
Plasmids		
pKO2.0	In-frame deletion plasmid	[44]
pBBR1MCS	4.5 kb γ R6K, <i>mob</i> RP4 <i>sacB</i> Gm ^R <i>lacZ</i> Cm ^R <i>lacZ</i>	[45]

Analytical methods

Mn(III) (10 mM), Mn(IV) oxides (5 mM), Fe(III) citrate (50 mM) and Fe(III) oxides (50 mM) were added from stock solutions prepared as described above. Mn(III) was monitored spectrophotometrically by absorbance measurement at 480 nm after filtering culture supernatants through 0.2 μ m pore-sized filters [39]. Mn(IV) oxide was monitored spectrophotometrically by absorbance measurement at 424 nm after reacting culture samples with a 2 mM benzidine solution [39]. Fe(III) reduction was monitored by measuring HCl-extractable Fe(II) with ferrozine [46]. NO_2^- was measured spectrophotometrically with sulfanilic acid-N-1-naphthyl-ethylene-diamine dihydrochloride solution. Anaerobic growth on TMAO, DMSO and fumarate was monitored by cell growth with absorbance at 600 nm.

In-frame gene deletion mutagenesis and genetic complementation analyses

Targeted genes were deleted from the *S. oneidensis* genome via application of an in-frame gene deletion system developed in our lab [44]. Regions corresponding to ~750 bp upstream and downstream of each gene were PCR-amplified (primers D1-D2 and D3-D4, Table S2) and subsequently joined using overlap-extension PCR (primers D1-D4, Table S2). Primers for each gene deletion are listed in Table S2. The resulting fragment was cloned into suicide vector pKO2.0, which does not replicate in *S. oneidensis*, and mobilized into wild-type MR-1 via conjugal transfer from *E. coli* donor strain β 2155 λ pir. *S. oneidensis* strains with the plasmid integrated into the genome were selected on solid LB medium containing gentamycin (15 μ g mL⁻¹). Single integrations were verified via PCR with primers flanking the recombination region (TF-TR) and were resolved from the genomes by plating on solid LB medium containing sucrose (10% w/v) with NaCl

omitted. In-frame deletions were verified by PCR. Primers for each gene deletion are listed in Table 2.2.

Table 2.2 Primers used in this study

Primer Name	Primer Sequence
<i>ΔfdnG</i>	
TF	TCCGCCACTGAAACATCAAATCACTTCA
TR	TCATGCCTGCTGCGATAACGAGTG
D1	GACTGGATCCTGGAAGGTAAACAACAGGTATTGGCA
D2	ATCGTCATTCGGATTCGTGTGAAGGGTTGCAGTGCTTTGGACTTCACC
D3	GGTGAAGTCCAAAGCACTGCAACCCTTCACACGAATCCGAATGACGATGAC
D4	GACTGTCTGACGGTGGCAAGGTAGCGTAATGGCAAGAAT
<i>ΔRTX</i>	
D1	GACTGGATCCCCTTTGCTACTTTGTCA
D2	AGCTTGCCAGATGGATGGTTAGAACCTTATCTAAGGTGGCTTCGACTTGCTC
D3	GAGCAAGTCGAAGCCACCTTAGATAAGGTTCTAACCATCCATCTGGCAAGCT
D4	GACTGTCTGACCCGCTTCAATGGTGTC
TF	CAGACCCTGCATTTGATGGTGTTGAT
TR	CCCATTGTAACTGAGTGCGGTAAACC

Results and discussion

Design of mutant screening technique and optimization of media composition

Screening techniques often include the addition of the desired electron acceptor to growth media, in which color changes directly or indirectly indicate the transformation of the compound of interest [29, 43]. Mn(III)-pyrophosphate solutions are red in color, with absorbance at 480 nm that changes proportionally to the concentration of Mn(III) [39]. Mn(II) appears clear, and interaction with carbonate or phosphate ions results in a white Mn(II) precipitate. The redox-dependent color changes facilitate identification of Mn(III) reduction activity by color changes from red to clear and can be employed to visually inspect large numbers of mutagenized colonies in microtiter plate wells for the Mn(III) reduction activity (Figure 2.4), and subsequently confirm the activity in batch liquid culture. In this work, random point mutations were induced in *S. oneidensis* cells, which were screened in liquid cultures with 10 mM Mn(III) in sterile 96 well-plates. Putative mutants were selected for the Mn(III) reduction-deficient phenotype based on the inability to produce clear solutions or white precipitates during anaerobic incubation with Mn(III) as electron acceptor.

Effect of growth media components

Wild-type and a previously isolated Mn(III) reduction-deficient mutant Mn3-1 were incubated in semi-defined and minimal media to compare Mn(III) reduction rates (Figure 1). Wild-type rates were 5X faster in semi-defined media with yeast extract compared to minimal media with no added organic compounds (except lactate as electron

donor). Mutant Mn3-1 was unable to reduce Mn(III) in minimal media, but reduced Mn(III) in semi-defined media at 20% wild-type levels.

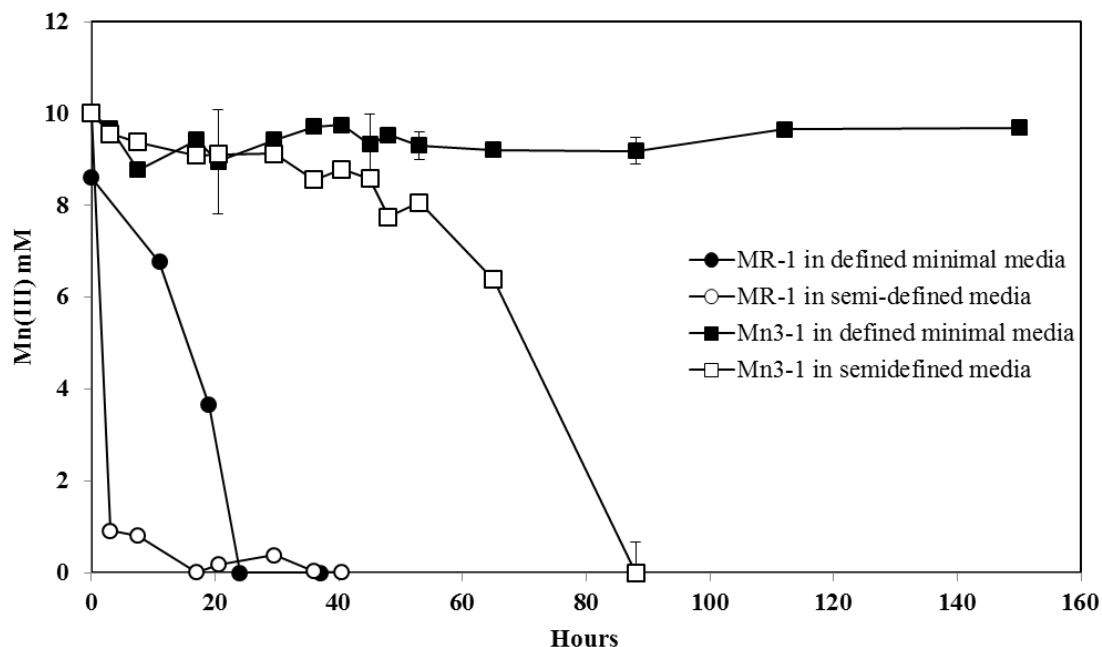


Figure 2.1. Differences in Mn(III) reduction rate due to media composition. Mn(III) reduction in minimal media incubations of *S. oneidensis* (●) and mutant Mn3-1 (■) and semi-rich media incubations of *S. oneidensis* (○) and mutant Mn3-1 (□) with lactate as electron donor.

Media-dependent rate differences may be the result of enhanced growth rates or, alternatively, to the abiotic reduction of Mn(III) by sulfides or thiol groups generated during growth on semi-defined media. Incubations of wild-type and mutant strains grown in and subsequently resuspended in cell-free supernatants of the wild-type strain indicated that wild-type supernatants restored Mn(III) reduction activity to Mn3-1 at near

wild-type rates (Figure 2.2 A). Supernatants alone did not reduce Mn(III) in 10 h incubations, and identical experiments with cells grown in minimal media did not display the supernatant-restored phenotype (data not shown). In addition, rates of aerobic growth and anaerobic NO_2^- reduction indicated that the wild-type and Mn3-1 mutant strains displayed growth and reduction rates (Figure 2.3), while normalization of rates to protein levels also resulted in the same reduction patterns (Figure 2.2 B). Therefore, anaerobic growth of *S. oneidensis* in rich media generates extracellular reducing metabolites (potentially, thiol compounds) that have the potential to catalyze abiotic Mn(III) reduction and produce false negative results. Mn(III) reduction experiments were therefore carried out exclusively on minimal media.

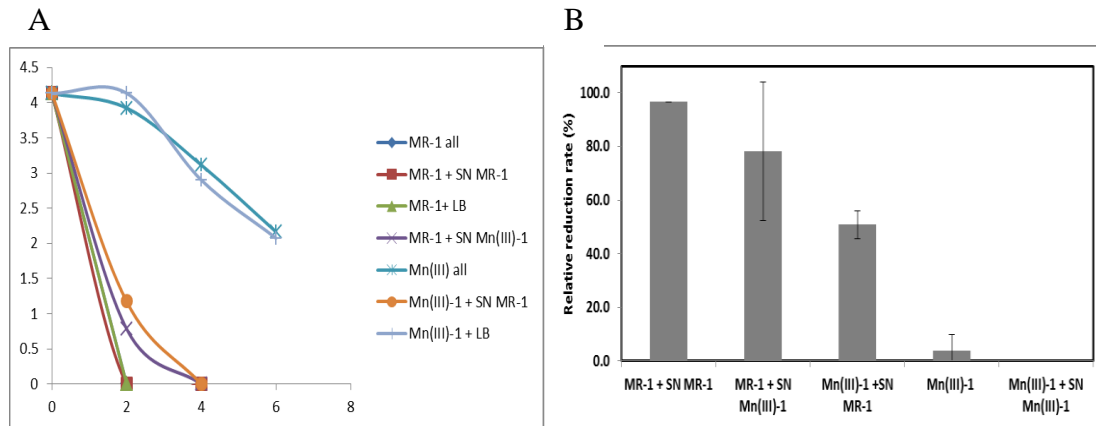
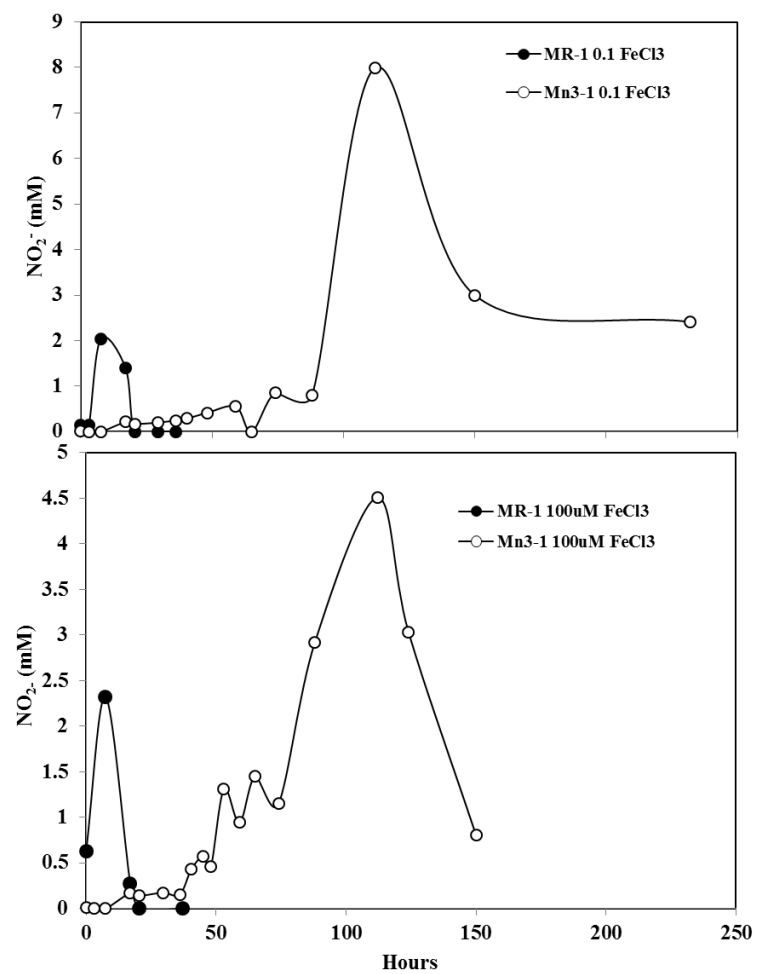


Figure 2.2. Effect of media composition on Mn(III) reduction by wild-type and Mn(III) reduction-deficient mutant Mn3-1. A) Rates of Mn(III) reduction and B) Mn(III) reduction rates normalized to wild-type levels of cell suspensions of wild-type *S. oneidensis* and mutant Mn3-1, resuspended in either MR-1 spent supernatant (SN MR-1) or Mn3-1 spent supernatant (SN Mn(III)-1) of cultures grown in LB.

Effect of iron

Nutrient iron in the form of FeCl_3 is added to M1 growth medium at concentrations of 100 μM . Nutrient Fe is required because Fe is present in the large pool of *c*-type cytochromes, which may be required for Mn(III) reduction. Two concentrations of FeCl_3 were tested to determine the optimum concentration during anaerobic Mn(III) reduction experiments. Results showed that, FeCl_3 concentration differences did not result in differences in NO_3^- reduction rates (Figure 2.4 A). NO_2^- peaks showed the same lag and produced approximately the same amount of NO_2^- for wild-type, and for the mutant strain. Decreased NO_2^- production in incubations with 100 μM FeCl_3 is most likely due to having missed the time point of maximum NO_2^- production. Mn(III) reduction rates, however, are directly proportional to FeCl_3 concentrations (Figure 2.4 B). Iron is a necessary nutrient during metal reduction because it is present in heme groups of cytochromes. Cytochromes in the metal reduction pathway have 4 or 10 heme groups per molecule. Fe limitation in the media may therefore limit the number of OM cytochromes necessary for Mn(III) reduction. Nitrate and nitrite reductases, however, are also cytochromes and require iron as components. The differences, therefore, may not be due to cytochrome content. Alternatively, these results suggest that Fe(III) may be acting as a shuttle during Mn(III) reduction.



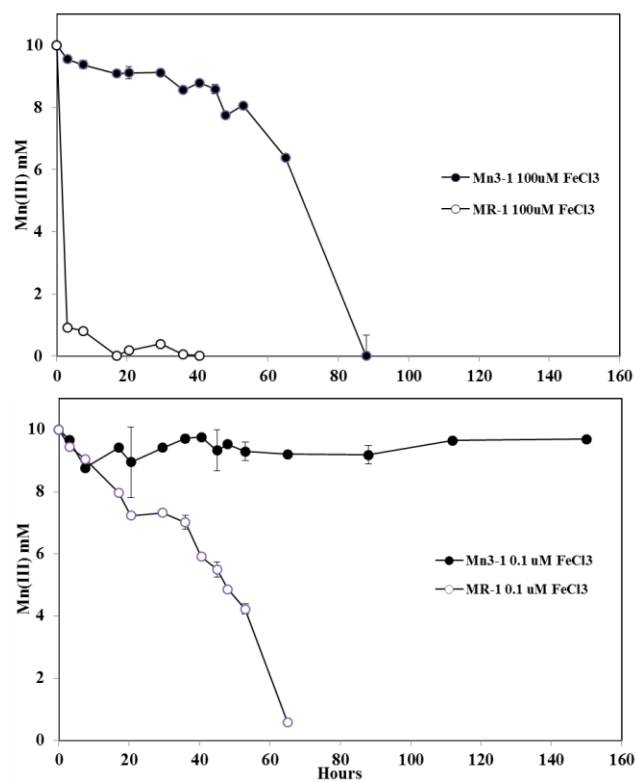


Figure 2.3. Effect of FeCl₃ additions on nitrite and Mn(III) reduction by *S. oneidensis*. A) NO₂⁻ and B) Mn(III) reduction rates of *S. oneidensis* MR-1 (●) and mutant Mn3-1 (○).

Identification of Mn(III) reduction-deficient mutant strains

5000 mutagenized colonies grown in LB agar plates were picked at random and inoculated individually in microtiter plate wells with 200 μ L of M1 media. Media was also amended with lactate (18 mM) as electron donor, and Mn(III)-pyrophosphate (10 mM) as electron acceptor. Incubations were conducted in an anaerobic chamber and visually inspected for the presence of red-colored Mn(III)-pyrophosphate. After 48 hours, 5 colonies remained red, indicating an inability to reduce Mn(III) (putative Mn(III) reduction-deficient mutants). The mutants were further tested for overall respiratory activity to determine the specificity of the mutation. The putative Mn(III) reduction-deficient mutants were tested for their ability to grow anaerobically on the terminal electron acceptors nitrate, nitrite, sulfite, thiosulfate, TMAO, fumarate, and solid and soluble forms of Mn(IV), Mn(III), and Fe(III) (Table 2.3). Mutant Mn3-1 was also impaired in DMSO, fumarate and solid Fe(III) oxide reduction. Interestingly, Mn3-1 displayed the ability to reduce soluble Fe(III) citrate, but did not reduce solid Fe(III) oxide. Such a mutant phenotype, displaying a distinction between solid and soluble Fe(III), has never been reported in *Shewanella* strains. Mn3-1 was therefore selected for further analysis to determine the identity of the genes responsible for the observed mutant phenotype. Mutants Mn3-2 to Mn3-5 were able to grow with O₂, fumarate, and DMSO, indicating that the mutant phenotypes were different than Mn3-1, and potentially contained a mutation in a central branch point of the anaerobic respiration pathway. Mutants Mn3-3, Mn3-4, and Mn3-5 were deficient in NO₃⁻ reduction, suggesting that the mutation was not specific for Mn(III) reduction. Mn3-2 was able to reduce NO₃⁻, and

further phenotypic characterization of Mn3-2 is required to determine if the mutation is metal reduction-specific.

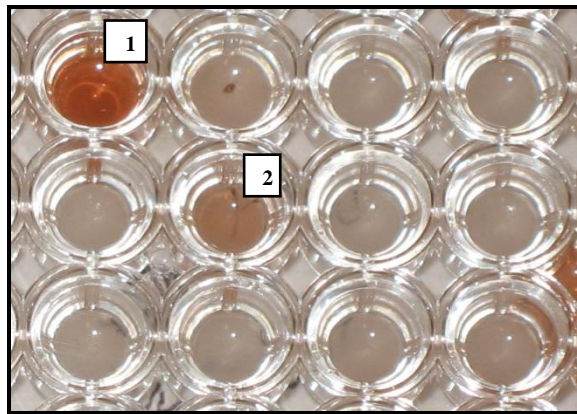


Figure 2.4. Representative image of Mn(III) reduction deficient mutant screens. An Mn(III) reduction-deficient mutant (1, top left), a mutant with impaired Mn(III) reduction (2, center) and wild-type phenotype (clear wells).

Table 2.3. Characterization of Mn(III) reduction-deficient mutants based on their ability (+) or inability (-) to reduce electron acceptors. +/- indicates partially impaired ability.

Strains	O ₂	MnO ₂	Mn(III)-pp	NO ₃ ⁻	Fe-cit	NO ₂ ⁻	DMSO	TMAO	Fumarate	SO ₃ ²⁻	FeOOH	S ₂ O ₃ ²⁻
MR-1	+	+	+	+	+	+	+	+	+	+	+	+
Mn3-1	+	+	-	+	+	+	-	+	-	+	-	+
Mn3-2	+		-	+	-		+		+			
Mn3-3	+		-	-	-		+		+/-			
Mn3-4	+		-	-	-		+		+/-			
Mn3-5	+		-	-	-		+					

Location of the point mutation in mutant Mn3-1

The Mn3-1 genome was sequenced and compared to the wild-type genome to identify locations of the point mutation. Genome sequencing of mutant Mn3-1 revealed that the mutant strain carried more than one point mutation. Table 2.4 lists the mutated genes identified by comparison with the annotated genome from *S. oneidensis* MR-1 in the NCBI database. The genes that showed an amino acid change were prioritized as initial candidates to be tested. FdnG codes for a nitrate-dependent formate dehydrogenase. RTX is an extracellular protein, and GspH participates in protein secretion. Previous studies have shown that GspD, the multimeric channel-forming secretin of T2SS is required for Fe(III) and Mn(III) reduction [24]. The involvement of FdnG, RTX and GspH genes in Mn(III) reduction was therefore tested. RTX and FdnG were deleted in frame (to generate mutants Δ RTX and Δ FdnG) and GspH was tested by complementation of mutant M3-1 with pBBR-1MCS with the wild type copy of *gspH*. Δ RTX and Δ FdnG were tested in incubations with Mn(III) as electron donor and formate and H₂ as electron acceptor. No differences in Mn(III) reduction activity between the wild-type and Mn3-1 mutant strain were observed (Figure 2.4). RTX toxins are a family of proteins found commonly in pathogenic strains, such as enterohemorrhagic *E. coli* [47]. RTX toxins are typically excreted into host cells. *S. oneidensis* RTX is homologous to haemolysin, involved in scavenging iron from host cells in pathogenic bacterial strains. Δ RTX was therefore also tested with Fe(III) citrate as electron acceptor. Growth curves showed that RTX is also not required for Fe(III) citrate reduction. RTX may play a scavenging role for Fe in the environment, perhaps under low concentrations, but this hypothesis needs to be further explored. Other hypothetical proteins listed on Table 2.4

should be the next genes tested for involvement in Mn(III) reduction by in-frame gene deletion mutagenesis and testing of the resulting mutants for Mn(III) reduction activity.

In summary, the newly developed rapid screening technique facilitated identification of several Mn(III) reduction-deficient mutant strains that were characterized for their overall respiratory capabilities with a suite of electron acceptors. Strain Mn3-1 displayed a distinction between solid and soluble Fe(III) reduction activity, a unique phenotype that has not been previously observed in *S. oneidensis*. Although the identity of the gene responsible for the Mn(III) reduction deficiency has not been obtained, none of the mutated genes belong to the Mtr pathway, suggesting that the mutations reside in genes encoding proteins other than those of the conventional Fe(III) and Mn(IV) reduction pathways. Strains M3-2, Mn3-3, Mn3-4 and Mn3-5 also showed Mn(III) reduction-deficient mutant phenotypes. Further characterization is needed, but Mn3-2 is the only strain that show a metal reduction-specific mutation.

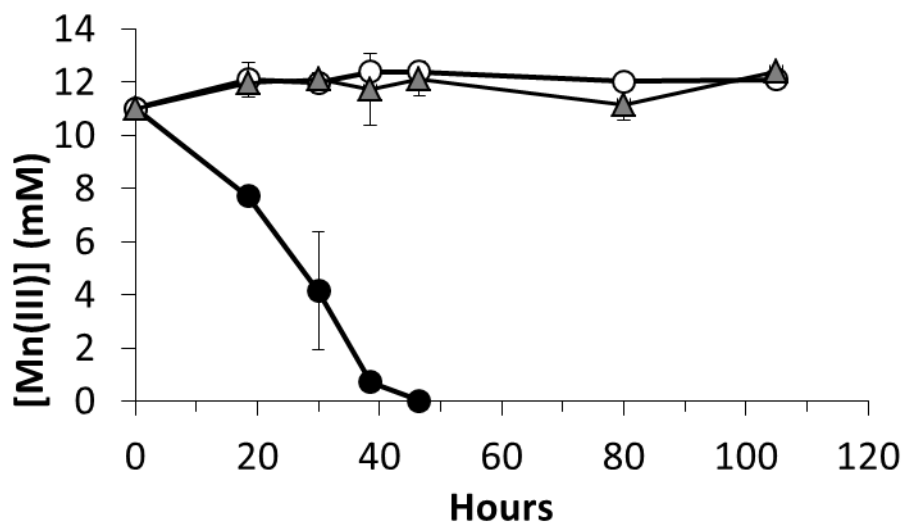


Figure 2.5. Complementation of Mn3-1 with a wild-type copy of *gspH*. Mn(III) reduction rates of *S. oneidensis* (●) and mutant Mn3-1 complemented with an empty vector (▲) or a vector containing a wild-type copy of *GspH* (○) with lactate as electron donor.

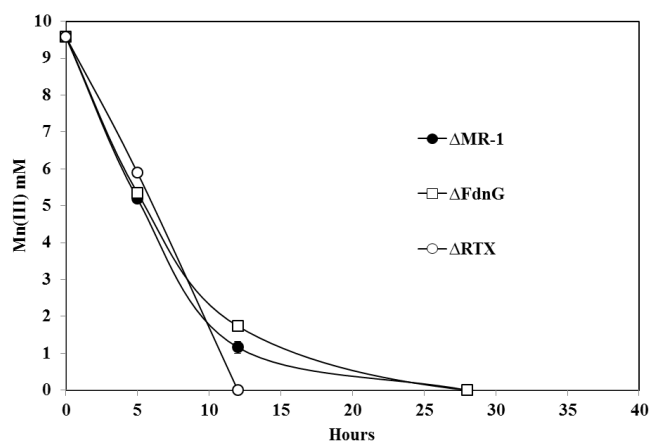


Figure 2.6. Mn(III) reduction rates of wild-type and in-frame deletion mutants. Mn(III) reduction rates of *S. oneidensis* (●) and mutant ΔRTX (○) and Δ*fdnG* (□) with formate as electron donor.

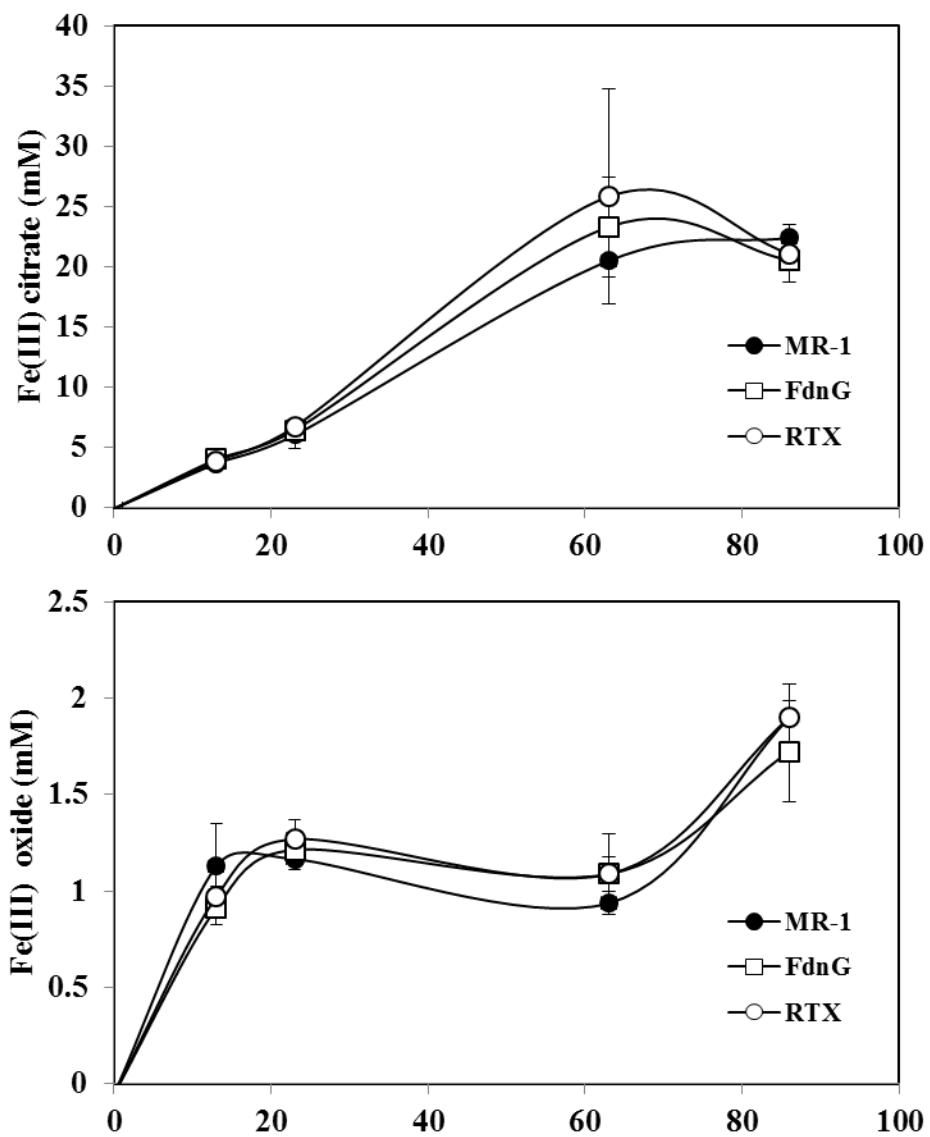


Figure 2.7. Fe(III) reduction rates of wild-type and in-frame deletion mutants. Fe(III) reduction rates of *S. oneidensis* (●) and mutant Δ RTX (○) and Δ fdnG (□) with formate as electron donor.

Table 2.4. Genes in mutant Mn3-1 containing mutated nucleotides compared to wild-type *S. oneidensis* MR-1.

gene/protein ID	WT (aa ⁺)	WT Codon	WT (N ⁺)	Mn3 (N)	Mn3 codon	Mn3 aa	Aa type change
NP_715743.1: selenium-containing formate dehydrogenase, nitrate inducible, alpha subunit	W	Tgg	T	G	ggg	G	nonpolar to polar
NP_715812.1: general secretion protein H	G	Gga	G	A	aga	R	polar to basic
NP_719678.1: RTX toxin	A	Gca	G	A	aca	T	nonpolar to polar
NP_719842.1: RTX toxin	M	Atg	A	G	gtg	V	chequea esto
NP_719051.1: hypothetical protein+1	H	taC	G	A	ata	Y	basic to polar
NP_715936.1: integral membrane domain-containing protein	S	tCc	G	A	aag	F	polar to nonpolar
NP_717150.1: hypothetical protein	R	agG	G	C	agc	S	basic to polar
NP_717799.1: hypothetical protein	A	Gcg	C	A	cgc	S	nonpolar to polar
NP_717466.1: hypothetical protein	M	aTg	T	G	agg	R	start to other
P_716695.1: hypothetical protein	A	Gca	G	C	cca	P	same
NP_715927.1: 3-dehydroquinase synthase	R	cGc	G	A	cac	H	same
NP_717248.1: surface antigen	S	Agt	A	G	ggt	G	same
NP_717277.1: phenylalanine 4-monooxygenase	D	gAt	A	C	gct	A	acid to non polar
NP_783499.1: carbon starvation protein A	stop	Taa	T	C	caa	Q	stop
flagellar regulatory protein A	tgG	G	C	G	acc	T	same
NP_717555.1: sodium:dicarboxylate symporter family	T	ccA	T	C	ggg	G	same
NP_718070.1: aminotransferase	G	Ggc	G	A	agc	S	same
NP_718509.1: prophage LambdaSo, host specificity protein J	A	Ccg	G	T	Acg	T	non polar to polar
NP_715726.1: HAD-superfamily hydrolase	V	Gtg	C	T	tac	M	same

*nucleotide in capital letters correspond to nucleotide mutated

⁺aa, aminoacid; N, nucleotide

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CHAPTER 3

ELECTRON TRANSPORT AND PROTEIN SECRETION PATHWAYS INVOLVED IN MN(III) REDUCTION BY *SHEWANELLA ONEIDENSIS* MR-1

[Chapter 3 has been submitted for publication in *Environmental Microbiology Reports*,
and is currently under review]

Summary

Soluble Mn(III) represents an important yet overlooked oxidant in marine and freshwater systems. The molecular mechanism of microbial Mn(III) reduction, however, has yet to be elucidated. Extracellular reduction of insoluble Mn(IV) and Fe(III) oxides by the metal-reducing γ -proteobacterium *Shewanella oneidensis* involves inner (CymA) and outer (OmcA) membrane-associated *c*-type cytochromes, the extracellular electron conduit MtrCAB, and GspD, the secretin of type II protein secretion. CymA, MtrCAB, and GspD mutants were unable to reduce Mn(III) and Mn(IV) with lactate, H₂, or formate as electron donor. The OmcA mutant reduced Mn(III) and Mn(IV) at near wild-type rates with lactate and formate as electron donor. With H₂ as electron donor, however, the OmcA mutant was unable to reduce Mn(III), but reduced Mn(IV) at wild-type rates. Analogous Fe(III) reduction rate analyses indicated that other electron carriers compensated for the absence of OmcA, CymA, MtrCAB, and GspD during Fe(III) reduction in an electron donor-dependent fashion. Results of the present study demonstrate that the *S. oneidensis* electron transport and protein secretion components involved in extracellular electron transfer to external Mn(IV) and Fe(III) oxides are also required for electron transfer to Mn(III), and that OmcA is essential only for electron transfer to Mn(III) with H₂ as electron donor.

Introduction

Microbial manganese (Mn) transformations are crucial components in the biogeochemical cycling of Mn, carbon, and other elements [1]. Mn(IV) oxides often dominate the pool of terminal electron acceptors in anaerobic marine and freshwater sediments where Mn(IV)-reducing bacteria are found in high cell density, and Mn(IV) reduction-linked organic carbon oxidation can account for up to 90% of total carbon remineralization capacity [2]. Mn(II) has traditionally been considered the dominant product of microbial Mn(IV) reduction in marine and freshwater systems [3]. Recent voltammetric analyses, however, have indicated that Mn(III) can represent up to 90% of the total soluble Mn pool in the redox-stratified water column of marine basins and in porewaters of freshwater sediments [4-6]. The source of the Mn(III) pool is not well defined but may include microbial Mn(IV) reduction and Mn(II) oxidation reactions that proceed through Mn(III) as a transient intermediate [7-13]. Microbial Mn(III) redox transformations may therefore represent important yet overlooked redox processes in marine and freshwater systems.

Mn(IV) and Fe(III) oxides exist as sparingly soluble amorphous or crystalline (oxy)hydroxides at circumneutral pH [12, 14-18]. Metal-reducing bacteria such as the γ -proteobacterium *Shewanella oneidensis* are therefore required to transfer electrons to external Mn(IV) and Fe(III) oxides unable to contact inner membrane (IM)-localized electron transport chains [8, 12, 19]. To overcome this problem, *S. oneidensis* transfers electrons to external Mn(IV) and Fe(III) oxides via a variety of novel respiratory strategies including i) direct enzymatic reduction by metal-reducing *c*-type cytochromes

located on the cell surface or along extracellular nanowires [1, 20, 21] ii) reductive (Mn(IV)) or non-reductive (Fe(III)) oxide solubilization followed by electron transfer to the resulting metal-organic ligand complexes [22, 23], and iii) indirect reduction by endogenous or exogenous electron shuttles such as flavins and humic acids [24, 25].

S. oneidensis contains a branched electron transport chain that consists of IM-localized dehydrogenases, menaquinone, and CymA, a menaquinol-oxidizing *c*-type cytochrome that functions as a central branch point for electron transport to Mn(IV), Fe(III), nitrate (NO₃⁻), nitrite (NO₂⁻), dimethylsulfoxide (DMSO), and fumarate [26-28]. Electron transfer proceeds from CymA to soluble, periplasmic *c*-type cytochromes such as MtrA [29-31], and subsequently to outer membrane (OM)-localized metal and flavin reductase complexes composed of transmembrane β -barrel protein MtrB and decaheme *c*-type cytochromes MtrC and OmcA [22, 32-35]. The involvement of the Mn(IV)- and Fe(III)-reducing electron transport system (CymA, OmcA, and MtrCAB) in Mn(III) reduction has yet to be examined.

Type II protein secretion is required for OM localization of MtrC and for, potentially, other proteins required for Mn(IV) and Fe(III) oxide reduction by metal-reducing *Shewanella* [36-39]. The Type II protein secretion pathway involves ATP-dependent translocation of exoproteins through the OM secretin GspD [40]. The *S. oneidensis* genome also encodes Type I and Type V secretion systems [41]. In Type I secretion systems, TolC functions as a tripartite efflux pump to export antibacterial drugs, protein toxins, and heavy metals [42-46]. In *S. oneidensis*, TolC is required for reduction of the synthetic electron shuttle anthraquinone-1,6-disulfonate (AQDS), presumably to efflux the deleterious end-product of AQDS reduction, AH₂DS [47, 48]. The Type V

secretion system is encoded by SO3800, an autotransporter-like serine protease that impairs exopolysaccharide production, but not Mn(IV) or Fe(III) oxide reduction by *S. oneidensis* [49, 50]. The involvement of the Types I, II, and V secretion systems in Mn(III) reduction by *S. oneidensis* has yet to be investigated.

The main objective of the present study was to determine if the electron transport chain components and protein secretion systems required for extracellular electron transfer to Mn(IV) and Fe(III) oxides by *S. oneidensis* were also required for electron transfer to Mn(III). To achieve this objective, a set of markerless, in-frame deletion mutants lacking the genes encoding CymA, OmcA, MtrC, MtrA, MtrB, GspD, TolC, and SO3800 were constructed and tested for Mn(III), Mn(IV), and Fe(III) reduction activities in defined minimal medium with lactate, formate, or H₂ as electron donor.

Materials and methods

Growth medium preparation of terminal electron acceptors

Minimal (M1) growth medium used in all incubations consisted of basal salts (g/L: MgCl₂, 160; CaSO₄, 45; EDTA, 16; FeSO₄, 0.96), 1 mL/L trace element solution (g/L: H₃BO₃, 2.8; ZnSO₄, 0.24; NaMoO₄, 0.75; CuSO₄, 0.042; MnSO₄, 0.17), 100 µL/L metal supplement solution (g/L: CoSO₄ 0.141; NiCl₂, 0.198; NaCl, 0.058), phosphate buffer (g/L: KH₂PO₄, 30; K₂HPO₄, 66; pH 7), amino acids (arginine, serine, glutamic acid, 20 mg/L), FeCl₃ (100 µM), NH₄ (1.9 g/L), and NaHCO₂ (20 mM). Amorphous Mn(IV) oxides were synthesized by reaction of MnCl₂ with KMnO₄ and neutralization with

NaOH [51]. Mn(III) was prepared by solubilization of Mn(III)-acetate followed by complexation with excess sodium pyrophosphate [11]. Poorly crystalline Fe(III) oxides were synthesized by neutralizing a solution of FeCl₃ with NaOH to pH 7 [52], and washing twice with M1 media. Soluble Fe(III) citrate was prepared by neutralizing an Fe(III) citrate solution with NaOH [53].

Table 3.1a. Strains used in this study

Strain	Features	Source
<i>S. oneidensis</i>		
MR-1	Wild-type strain	ATCC
$\Delta cymA$	In-frame <i>cymA</i> deletion mutant	This study
$\Delta mtrA$	In-frame <i>mtrA</i> deletion mutant	This study
$\Delta mtrB$	In-frame <i>mtrB</i> deletion mutant	[54]
$\Delta mtrC$	In-frame <i>mtrC</i> deletion mutant	This study
$\Delta omcA$	In-frame <i>omcA</i> deletion mutant	This study
$\Delta mtrC\Delta omcA$	In-frame <i>mtrC</i> and <i>OmcA</i> deletion mutant	This study
$\Delta tolC$	In-frame <i>tolC</i> deletion mutant	This study
$\Delta gspD$	In-frame <i>gspD</i> deletion mutant	This study
$\Delta SO3800$	In-frame SO3800 deletion mutant	[55]
<i>E. coli</i>		
$\beta 2155 \lambda$ pir	<i>thrB1004 pro thi strA hsdS lacZ_M15</i> (F9 <i>lacZ</i> Δ <i>M15 lacIq traD36 proA1 proB1</i>) Δ <i>dapA::erm pir::RP4</i> Km ^R	[56]
EC100D pir-116	<i>F- mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) $\phi 80\Delta$ <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74 recA1 endA1 araD139</i> Δ (<i>ara, leu</i>)7697 <i>galU galK</i> λ - <i>rpsL nupG pir</i> - 116(DHFR)	Epicentre

Table 3.1b. Plasmids used in this study

Plasmids	Features	Source
pKO2.0	In-frame deletion plasmid	[55]
pBBR1MCS	4.5 kb γ R6K, <i>mob</i> RP4 <i>sacB</i> Gm ^R <i>lacZ</i> Cm ^R <i>lacZ</i>	[54]

In-frame gene deletion mutagenesis and genetic complementation analyses

Targeted genes were deleted from the *S. oneidensis* genome via application of an in-frame gene deletion system [57]. Regions corresponding to ~750 bp upstream and downstream of each gene were PCR-amplified (primers D1-D2 and D3-D4, Table 3.2) and subsequently joined using overlap-extension PCR (primers D1-D4, Table 3.2). The resulting fragment was cloned into suicide vector pKO2.0, which does not replicate in *S. oneidensis* and mobilized into wild-type MR-1 via conjugal transfer from *E. coli* donor strain β 2155 λ pir. *S. oneidensis* strains with the plasmid integrated into the genome were selected on solid LB medium containing gentamycin (15 μ g mL⁻¹). Single integrations were verified via PCR with primers flanking the recombination region (TF-TR) and were resolved from the genomes by plating on solid LB medium containing sucrose (10% w/v) with NaCl omitted. In-frame deletions were verified by PCR. Genetic complementation of the targeted genes was carried out by cloning the wild-type gene into broad-host-range cloning vector pBBR1MCS [58] and conjugally transferring the recombinant vector into the respective mutant via bi-parental mating procedures [48].

Table 3.2. Primers used in this study

Primer Name	Primer Sequence
<i>ΔcymA</i>	
D1	GACTGGATCCGGCAGGTAACGCTAAAATGCCCA
D2	ACCTTATCCTTTTGGATAGGGGTGAGCTGCACGCCAGTTCATTACTCTATCTCC
D3	GGAGATAGAGTAATGAACTGGCGTGCAGCTCACCCCTATCCAAAAGGATAAGGT
D4	GACTGTCGACGGGTTTTGCAGTGATTGGTAAGGGAG
TF	GCGGTTTGAATCGCCCAACTTGAG
TR	CTCAGCAAGCGATAATCGAGTTACCACG
<i>ΔmtrA</i>	
D1	GACTGGATCCCCCTTTGCTACTTTGTCA
D2	AGCTTGCCAGATGGATGGTTAGAACCTTATCTAAGGTGGCTTCGACTTGCTC
D3	GAGCAAGTCGAAGCCACCTTAGATAAGGTTCTAACCATCCATCTGGCAAGCT
D4	GACTGTCGACCCGCTTCAATGGTGTC
TF	CAGACCCTGCATTTGATGGTGTTGAT
TR	CCCATTGTAAGTGAAGTGCGGTAAACC
<i>ΔmtrC</i>	
D1	GACTGGATCCAGTGAAGGCCATTATCTGAAATATGCAGCG
D2	TTAGTGTGATCTGCAACTGTTGGGGTCATTGTGACGGACTTGCTGCGA
D3	TCGCAGCAAGTGCCGTCACAAGACCCCAACAGTTGCAGATCACACTAA
D4	GACTGTCGACCGCTAGGCTTGTTAAGATCGGAATCTGT
TF	GCTAAGTGTTTCAGGTGCCATAACAAGGAA
TR	GCGTTTTTCGGCGTGACAGG
<i>ΔomcA</i>	
D1	GACTGGATCCGGTCAATGTCAGCTCTGCCACAA
D2	GATGGAGTATGGCACGTTGCACAGGCACCCAACATCGCTTTTGTG
D3	CAACAAAAGCGATGTTGGGTGCCTGTGCAACGTCCCATACTCCATC
D4	GACTGTCGACCTAATTTACCCGCAGCAGAAACAACGT
TF	CTTAGTGGATTGCAGCACCGAACTG
TR	CGTGGCAAGAAGCACATACTTCGTG
<i>ΔtolC</i>	
D1	GACTGGATCCGTGCAAACCTTGTGCCTGTGATGAAT
D2	CAGCTTTTAGGCCGTTGTTGATGGCGGCTAGAGTTAATGCTACGCATAGAGAACG
D3	CGTTCTCTATGCGTAGCATTAACTCTAGCCGCCATCAACAACGGCCTAAAAGCTG
D4	GATCGTCGACGGATGCTTAATGGTCACTTCAGCCC
TF	CGTTATAGGCAGCCTCACGGTCTA
TR	CTAACTATGTGTCCCGTGCGAATGC
<i>ΔgspD</i>	
D1	GACTGGATCCCCTCATCTTCTCCTACGGCTTGGTCTC
D2	CGCATCTGAGTCTCTAACCCTTTGCCGCCC GCCACAACCTCCAGCAAT
D3	ATTGCTGGAGTTGTGGCGGGCGGCAAAGGGTTAGAGACTCAGATGCG
D4	GACTGTCGACCGGTTTGGCTTGAGCACTTCTTTGAGC
TF	GCATAAGCCATTGAGCCAAGTCGT
TR	CATTTGACGCAAAATCGCCCGTAGAC

Results and Discussion

Effects of electron donors on Mn(III) reduction by wild-type *S. oneidensis*

Microbial Mn(III) reduction may be an important yet overlooked terminal electron-accepting process in marine and freshwater environments [20, 21]. *S. oneidensis* reduces Mn(III)-pyrophosphate (designated below as Mn(III)) in minimal or rich growth media with lactate as electron donor [57]. The electron transport chain components required for microbial Mn(III) reduction, however, have not been identified. In addition, although the majority of metal reduction studies with *S. oneidensis* are carried out with lactate as electron donor, only a limited number of studies have determined metal reduction activity with other environmentally relevant electron donors such as formate and H₂ [59, 60]. In the present study, the Mn(III) reduction activity of *S. oneidensis* electron transport chain and protein secretion mutants was determined in defined minimal medium with lactate, formate, or H₂ as electron donor. To facilitate direct rate comparisons, the Mn(III), Mn(IV) oxide, Fe(III) citrate, and Fe(III) oxide reduction activities of the *S. oneidensis* wild-type and mutant strains were determined under identical culture conditions.

Mn(III) reduction activities were 4- and 5-fold greater with lactate and formate than with H₂ as electron donor (Fig. 3.1). In a similar manner, Fe(III) citrate reduction activities were 4-fold greater with lactate and formate than with H₂ as electron donor, while Fe(III) oxide reduction activities were similar for all three electron donors (Fig. 3.2). These results are similar to those reported for the electron donor-dependent reduction of Fe(III) citrate by membrane fractions of *S. oneidensis* [48] and whole cell suspensions of *S. putrefaciens* CN32 [11, 20, 21]. The previously reported differences in

Fe(III) citrate reduction activity were attributed to differences in electron donor solubility, with lactate and formate solubilities exceeding that of H₂ by approximately 20-fold [61]. Results of the present study indicate that electron donor solubility may affect Mn(III) reduction activity in a similar manner. Alternately, the activity of hydrogenases at the head end of the electron transport chain may limit metal reduction rates with H₂ as electron donor and Mn(III), Mn(IV), or Fe(III) as electron acceptor.

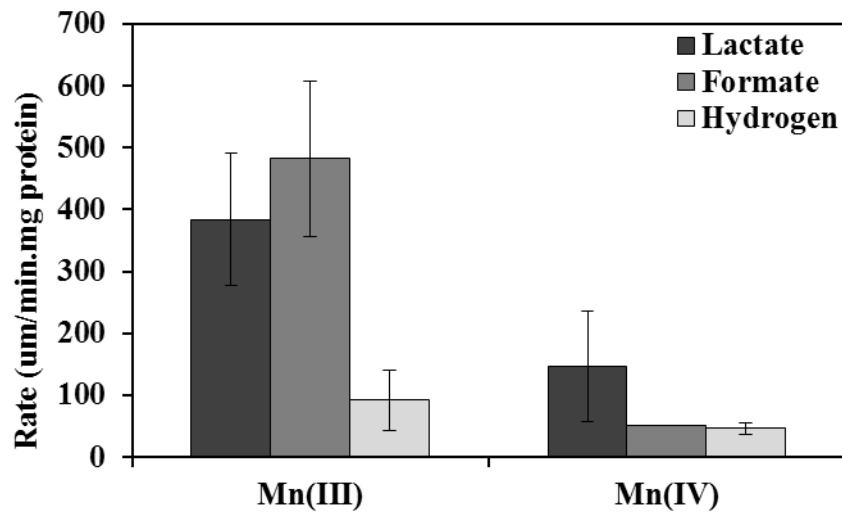


Figure 3.1. Mn(III) and Mn(IV) reduction activity of wild-type *S. oneidensis* during anaerobic incubations with lactate (■), formate (▒) or H₂ (□) as electron donor and Mn(III) (10 mM) or Mn(IV) oxide (5 mM) as electron acceptor.

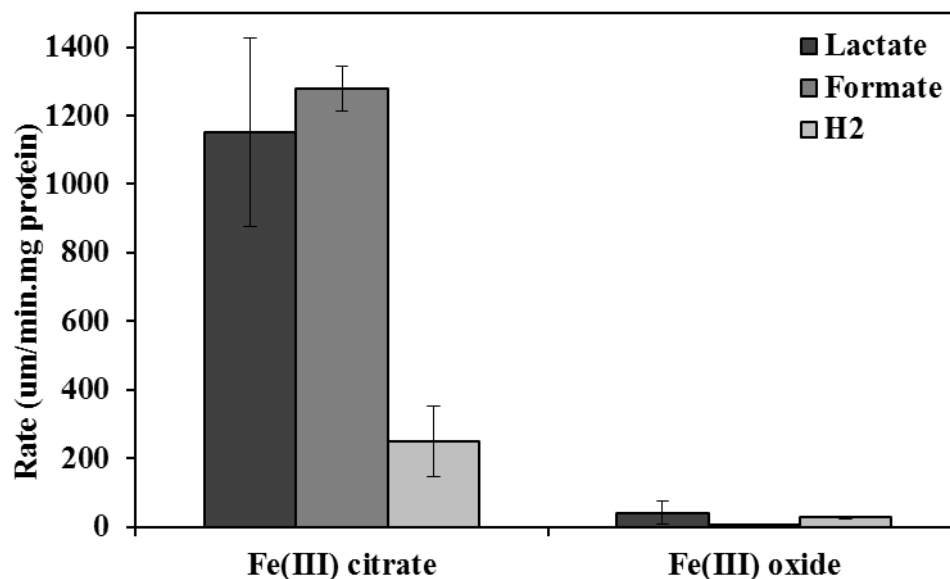


Figure 3.2 Relative rates of Fe(III) citrate and Fe(III) oxide reduction on wild-type *S. oneidensis*

Comparison of Mn(III) and Mn(IV) reduction activities of wild-type *S. oneidensis*

Recent voltammetric analyses indicate that Mn(IV) oxide reduction by *S. oneidensis* requires an initial one-electron reductive dissolution step that produces Mn(III) as a transient intermediate [35, 60, 62, 63]. Results of the present study indicate that the rate of Mn(IV) oxide reductive dissolution, rather than the rate of Mn(III) reduction, limits overall Mn(IV) oxide reduction activity: with lactate or formate as electron donor, Mn(III) reduction rates were approximately 5-to-10-fold greater than the corresponding Mn(IV) oxide reduction rates, while Mn(III) and Mn(IV) oxide reduction rates were nearly identical with H₂ as electron donor (Figure 3.1). Previous voltammetric analyses also indicated that Fe(III) oxide reduction by *S. oneidensis* may proceed through a non-reductive dissolution step with soluble organic-Fe(III) produced as a transient

intermediate [35] . Similar to Mn(IV) oxide reduction activity, the Fe(III) oxide reduction activity of *S. oneidensis* is not limited by rates of electron transfer to soluble organic-Fe(III): as previously reported [61] and confirmed in the present study (Figure 3.2), Fe(III) citrate reduction rates are approximately 20-fold greater than the corresponding Fe(III) oxide reduction rates with all three electron donors. Fe(III) oxide reduction activities of *S. putrefaciens* and *S. oneidensis* whole cells [61] and *S. oneidensis* cell membrane fractions [64] are limited by Fe(III) oxide surface area, with non-reductive Fe(III) dissolution overcoming this limitation by producing more readily reducible soluble organic-Fe(III) intermediates. Likewise, Mn(IV) oxide surface area may limit Mn(IV) oxide reduction activity by *S. oneidensis* with reductive Mn(IV) dissolution overcoming this limitation by producing more readily reducible soluble Mn(III) intermediates.

Mn(III) and Mn(IV) oxide reduction activities of *S. oneidensis* electron transport chain mutants

Previous studies demonstrated that CymA, MtrC, MtrA, and MtrB were required for Mn(IV) and Fe(III) oxide reduction by *S. oneidensis* [32, 33, 35]. In the present study, $\Delta cymA$, $\Delta mtrC$, $\Delta mtrA$, $\Delta mtrB$, and $\Delta mtrC\Delta omcA$ were also severely impaired in Mn(III) reduction activity, regardless of electron donor (Figure 3.3). These results indicate that the electron transport pathways terminating with reduction of Mn(III) overlap with those required for reduction of Mn(IV) and Fe(III) oxides, with CymA, MtrC, MtrA, and MtrB as common electron transport chain components.

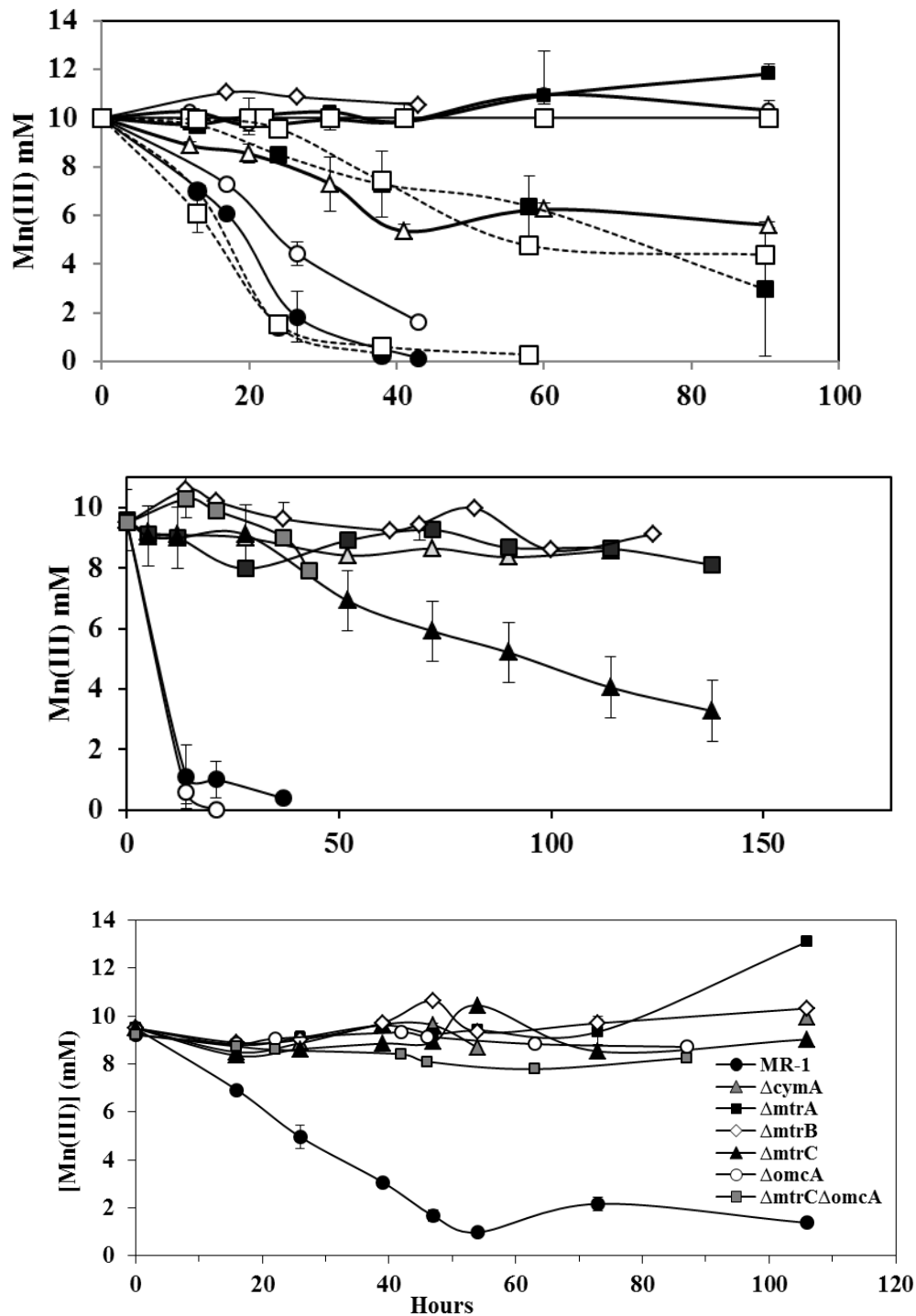


Figure 3.3. Mn(III) reduction activity of wild-type and electron transport chain mutants of *S. oneidensis*. Wild-type (●) and mtr mutants $\Delta cymA$ (▲), $\Delta mtrA$ (■), $\Delta mtrB$ (◇), $\Delta mtrC$ (▲), $\Delta omcA$ (○), and $\Delta mtrC\Delta omcA$ (◼) with A) lactate, B) formate, or C) H₂ as electron donor. Dashed lines correspond to mutant strains complemented with the corresponding wild-type genes.

Recent proteoliposome studies indicated that *S. oneidensis* MtrCAB forms an OM-spanning conduit that transfers electrons to external Fe(III) substrates [65]. The inability of $\Delta mtrC$, $\Delta mtrA$, or $\Delta mtrB$ to reduce Mn(III) suggests that MtrCAB also transfers electrons to external Mn(III). Extracellular electron transfer to soluble electron acceptors such as Mn(III) is not unprecedented in *S. oneidensis*: reduction of highly soluble DMSO is catalyzed by DMSO reductases located on the *S. oneidensis* OM, potentially alleviating problems associated with DMSO uptake and mutagenicity [66, 67]. In addition, Mn(II) is oxidized on the extracellular side of the exosporium of Mn(II)-oxidizing *Bacillus* sp. strain SG-1, perhaps as a strategy to avoid Mn(II) toxicity [64]. In a similar fashion, *S. oneidensis* may avoid problems associated with Mn(II) toxicity by reducing Mn(III) extracellularly.

Previous studies demonstrated that an *omcA* insertion mutant reduced soluble Fe(III) substrates at wild-type rates [68-73], but reduced Mn(IV) oxide at only 55% wild-type rates with lactate as electron donor [65, 74, 75](Table 3.3). Expression of wild-type OmcA in a mutant lacking MtrC, OmcA, and MtrF rescued Mn(IV) reduction to 53% wild-type rates, while Fe(III) citrate reduction was not rescued by OmcA expression in these same mutants [66, 67](Table 3.3). These observations led to the proposition that OmcA was involved in attachment to solid metal oxide surfaces [23, 76]. In the present study, $\Delta omcA$ reduced Mn(IV) oxides at wild-type rates with formate or H₂, and at 50% wild-type rates with lactate as electron donor (Figure 3.4). On the other hand, $\Delta omcA$ reduced Mn(III) at wild-type rates with lactate and formate as electron donor, but reduced Mn(III) at only 8% wild-type rates with H₂ as electron donor (Figure 3.3).

A previous study also reported that $\Delta omcA$ reduced Mn(IV) oxides at wild-type rates with lactate as electron donor, while $\Delta cymA$ and $\Delta mtrC\Delta omcA$ reduced Mn(IV) oxides at 41% and 85% wild-type rates, respectively, with lactate as electron donor [77] (Table 3.3). Results of the present study indicated that $\Delta cymA$ and $\Delta mtrC\Delta omcA$ were completely impaired in Mn(IV) oxide reduction activity (Figure 3.4, Table 3.3) and that $\Delta mtrC$ reduced Mn(IV) oxides and Mn(III) at 0% and 15% wild-type rates with formate and H₂, and at 25% and 54% with lactate as electron donor. Reasons for these discrepancies are unclear, but may be due to the relatively short incubation periods (< 48 h), high cell densities (10⁹ cells/mL), and low Mn(IV) oxide concentrations (300 μ M) used in the previous study [69]. In the present study, lower cell densities (10⁷ cells/mL), higher Mn(IV) oxide concentrations (5 mM), and longer incubation periods were employed to ensure that Mn(IV) reduction activity was not limited by short incubation times or low Mn(IV) oxide concentrations. The involvement of OmcA, MtrCAB, and GspD in Mn(III) and Mn(IV) reduction was confirmed via restoration of wild-type Mn(III) and Mn(IV) reduction activities to mutant transconjugates provided with wild-type copies of each gene on pBBR1MCS (Fig. 3.3 A). These results demonstrate that Mn(III) reduction by *S. oneidensis* involves the extracellular electron transport and protein secretion components (CymA, MtrCAB, and GspD) required for reduction of external Mn(IV) and Fe(III) oxides, and that OmcA is essential only for electron transfer to Mn(III) with H₂ as electron donor.

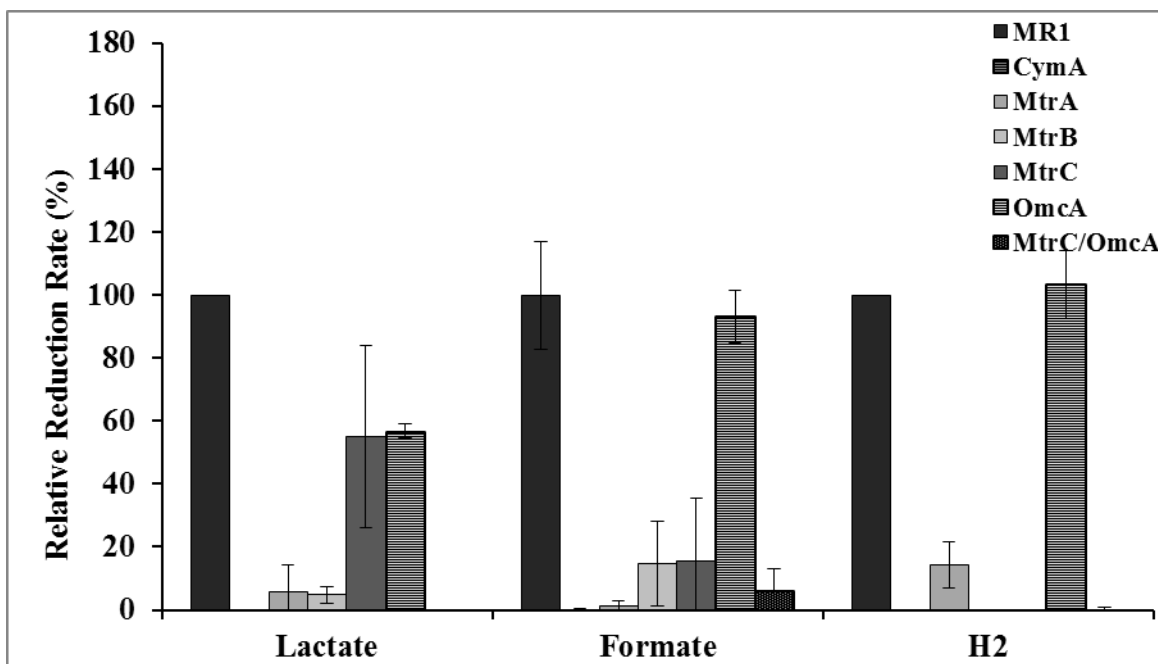


Figure 3.4. Mn(IV) reduction activity of wild-type *S. oneidensis* and electron transport chain mutants. *S. oneidensis* (■) $\Delta cymA$ (■) $\Delta mtrA$ (■), $\Delta mtrB$ (■), $\Delta mtrC$ (■), $\Delta omcA$ (■), and $\Delta mtrC\Delta omcA$ (■) with A) lactate, B) formate, or C) H_2 as electron donor.

Table 3.3. Reduction rates of Mn(IV), Fe(III) citrate and Fe(III) oxides relative to wild-type with lactate as electron donor

Metal reduction activity of mutant strains with lactate as electron donor ¹						
Electron acceptor	mM	OmcA	MtrC	MtrB	CymA	Reference
Mn(IV)	5	60% (90 h)	56% (140h)	5%	20% (60 h)	This study
	2.5	30% rescue	53% rescue	n/d ²	n/d	[78]
	0.3	wt (not shown)	wt (not shown)	0%	41% (24 h)	[79]
	5	55% (96 h)	25% (24 h) ¹	n/d	7% (96 h)	[71]
Fe(III) citrate	50	90% (90 h)	68% (40 h)	0%	60%* (94 h)	This study
	10	no rescue	50% rescue	n/d	n/d	[71]
	5	wt (6 h)	30% (6h)	n/d	n/d	[78]
	10	96% (96 h)	wt (not shown)	n/d	4% (96 h)	[71]
Fe(III) oxides	50	80% (150 h)	60% (150 h)	113%	60% (150 h)	This study
	5	70% (30 h)	40% (30 h)	n/d	n/d	[69, 70]
	20	n/d	n/d	<10%	0% (24 h)	[78]
	2	76% (96 h)	wt (not shown)	n/d	n/d	[79]

¹after lag period indicated in parentheses

²n/d, not determined

Fe(III) reduction activity of *S. oneidensis* electron transport chain mutants

Results of several previous studies have indicated that the Fe(III) reduction activity of $\Delta mtrC$ is not well constrained (Table 3.3). In an earlier study, $\Delta mtrC$ reduced Fe(III) citrate and Fe(III) oxide at wild-type rates with lactate as electron donor [69, 80], while in a more recent study $\Delta mtrC$ reduced Fe(III) citrate and Fe(III) oxide at only 30%-40% wild-type rates with lactate as electron donor [79] (Table 3.3). In the present study, $\Delta mtrC$ reduced Fe(III) citrate and Fe(III) oxide at 60% wild-type rates with lactate, formate, or H₂ as electron donor (Table 3.3). In agreement with previous findings with lactate as electron donor [71] (Table 3.3), $\Delta omcA$ reduced Fe(III) citrate and Fe(III) oxide at wild-type rates with lactate, formate, or H₂ as electron donor (Figure 3.6; Table 3.3). Several previous studies reported that $\Delta mtrA$ and $\Delta mtrB$ were severely impaired in Fe(III) citrate and Fe(III) oxide reduction activities with lactate as electron donor [69, 80] (Table 3.3). In the present study, $\Delta mtrA$ and $\Delta mtrB$ reduced Fe(III) oxide at wild-type rates with lactate and H₂ as electron donor after lag periods of 20 h and 60 h, respectively (Fig. 3.5). $\Delta mtrA$ also reduced Fe(III) citrate at wild-type rates with H₂ as electron donor after a 24 h lag period (Fig. 3.6). $\Delta mtrA$ was unable to reduce Fe(III) oxide or Fe(III) citrate with formate as electron donor even after extended incubation periods (Figure 3.5). In agreement with all previous studies, $\Delta mtrB$ was severely impaired in Fe(III) citrate reduction activity during extended incubation periods, regardless of electron donor (Figure 3.5). The relatively short 25 h incubation periods employed in the previous studies may have inadvertently missed the Fe(III) citrate and Fe(III) oxide reduction

activities detected during the extended incubation periods employed in the present study (Figure 3.5).

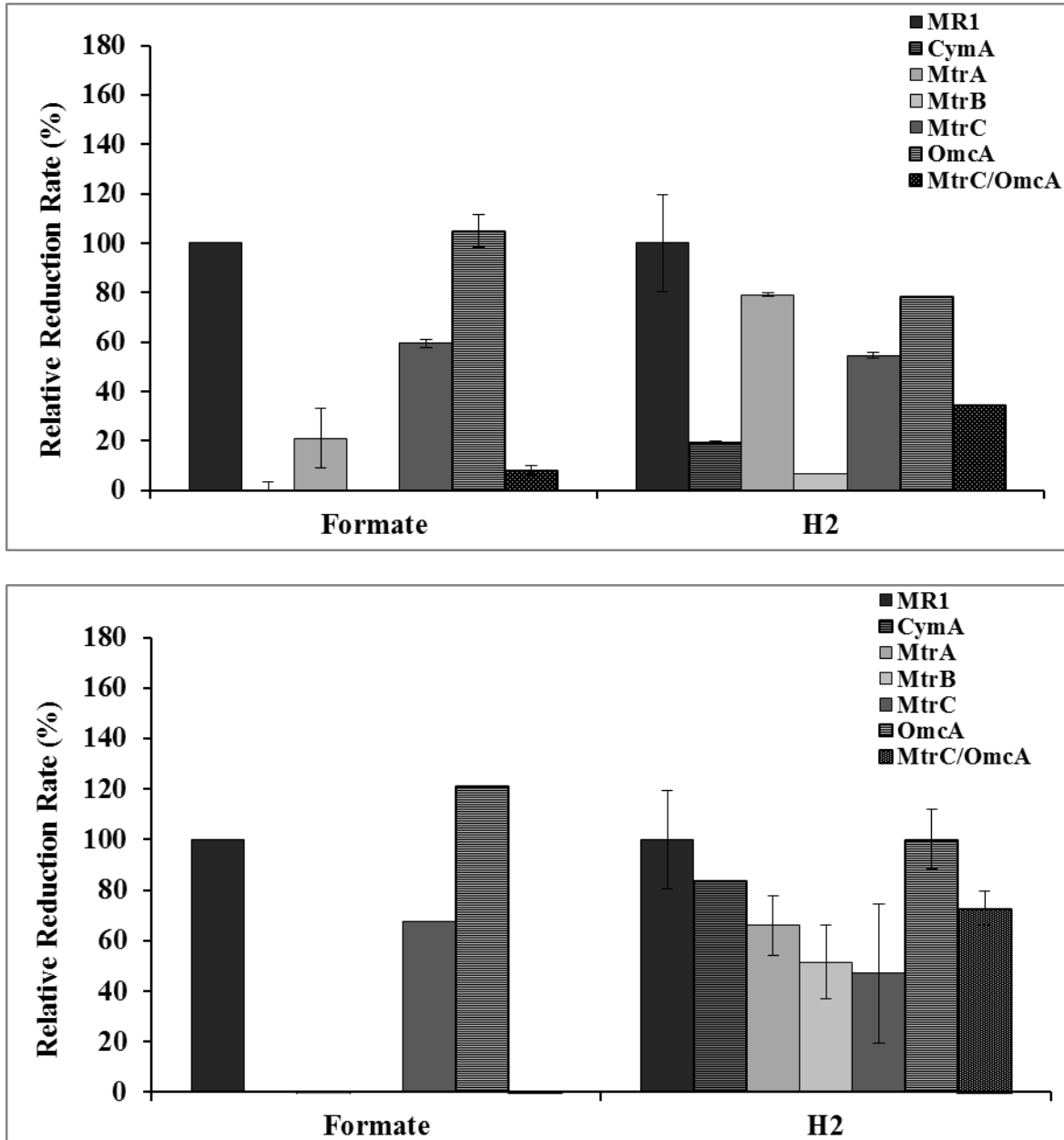


Figure 3.5. Metal reduction activity of *S. oneidensis* and electron transport chain mutants. *S. oneidensis* wild-type (■) and Mtr mutants $\Delta cymA$ (■), $\Delta mtrA$ (■), $\Delta mtrB$ (■), $\Delta mtrC$ (■), $\Delta omcA$ (■), and $\Delta mtrC\Delta omcA$ (■) with (A) Mn(III), (B) Mn(IV) oxide, (C) Fe(III) citrate, or (D) Fe(III) oxide as electron acceptor and lactate, formate or H₂ as electron donor normalized to wild-type levels.

Previous studies demonstrated that modularity of the *S. oneidensis* respiratory system provides compensation for the absence of CymA, MtrCAB, and OmcA during Fe(III) reduction with lactate as electron donor [69]. The MtrA paralogs MtrD, DmsE, and SO4360, for example, compensate for the absence of MtrA during Fe(III) citrate reduction, while the MtrB paralog MtrE compensates for the absence of MtrB [79]. Results of the present study indicate that the *S. oneidensis* respiratory system provides compensation for the absence of CymA, MtrCAB, and OmcA during Fe(III) reduction in an electron-donor-dependent fashion. However, other electron transport components are unable to compensate for the absence of CymA, MtrCAB, or GspD during Mn(III) or Mn(IV) reduction since the MtrCAB mutants were unable to reduce Mn(III) or Mn(IV) oxide during extended anaerobic incubation periods, regardless of electron donor (Figures 3.3 and 3.4). The modularity of the *S. oneidensis* respiratory system thus provides electron donor-dependent compensation for the absence of CymA, MtrCAB, and GspD only during Fe(III) reduction, and not during Mn(III) or Mn(IV) reduction.

Mn(III) reduction activity of Types I, II, and V protein secretion-deficient mutants of *S. oneidensis*

The Type I protein secretion mutant $\Delta tolC$ reduced Mn(III) at wild-type rates with lactate and formate, and reduced Mn(III) at rates 60% greater than wild-type with H₂ as electron donor (Figure 3.6). $\Delta tolC$ reduced Mn(IV) oxides, Fe(III) citrate, and Fe(III) oxides at wild-type rates, regardless of electron donor (Figures 3.7 A B C). Similarly, a

tolC transposon mutant also reduced Fe(III) oxide and Mn(IV) oxide at wild-type rates with lactate as electron donor [69]. Although TolC is involved in heavy metal and AH₂DS efflux in *E. coli* and *S. oneidensis*, respectively, results of the present study indicate that TolC is not required for Mn(III), Fe(III), or Mn(IV) reduction by *S. oneidensis*, regardless of electron donor. *ΔgspD*, on the other hand, was severely impaired in Mn(III) reduction activity and, contrary to previous studies [46, 65, 71-73], was also unable to reduce Mn(IV) oxide, regardless of electron donor (Figure 3.6 and 3.7A). Results of the present study thus indicate that Mn(III) reduction is linked to type II protein secretion. Since the type II protein secretion system is required for OM localization of MtrC [79], these results support the previous finding that *ΔmtrC* is also severely impaired in Mn(III) reduction activity.

The type V protein secretion mutant *ΔSO3800* reduced Mn(IV) oxide, Fe(III) oxide, and Fe(III) citrate at wild-type rates (Figure 3.7 A-C), and reduced Mn(III) at rates 45% greater and 75% greater than the wild-type strain with H₂ and formate as electron donor, respectively (Figure 3.6). Previous studies demonstrated that exopolysaccharides interfere with extracellular electron transfer by *S. oneidensis* [81], and that *ΔSO3800* was impaired in exopolysaccharide production and attachment to hematite surfaces [82]. With lactate as electron donor, *ΔSO3800* reduced hematite, Fe(III) oxide, Fe(III) citrate, and Mn(III) at wild-type rates [71]. The results on this study indicate that *SO3800* is not required for Mn(III) reduction by *S. oneidensis*, and that the increased rates of metal reduction by *ΔSO3800* may be due to decreased exopolysaccharide production.

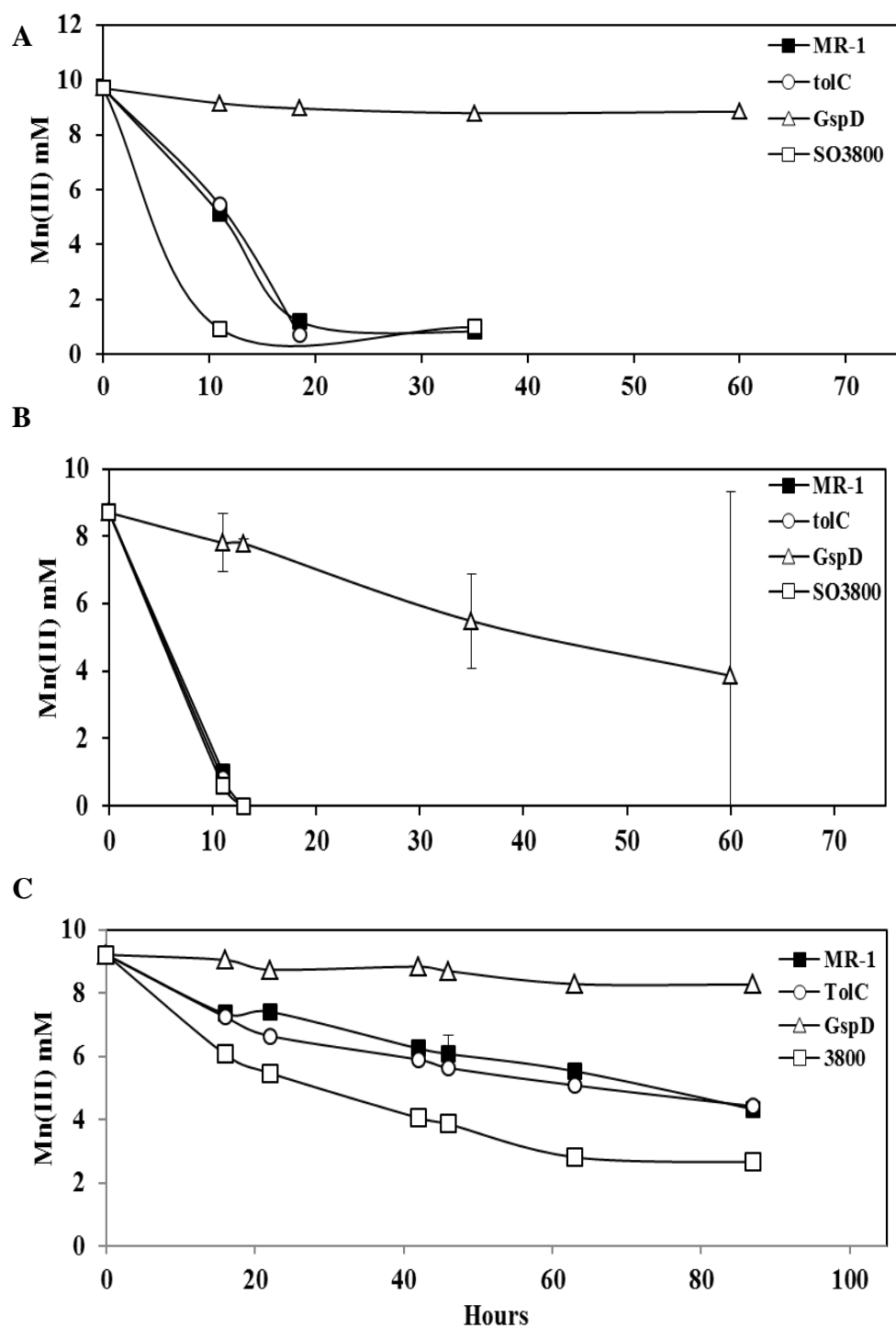


Figure 3.6. Mn(III) reduction activity of wild-type and protein secretion mutants of *S. oneidensis*. Wild-type *S. oneidensis* (■) and mutants $\Delta tolC$ (○) $\Delta gspD$ (△), $\Delta SO3800$ (□), with lactate (A), formate (B) or H_2 (C) as electron donor.

Results of the present study indicate that the *S. oneidensis* electron transport (CymA, MtrC, MtrA, and MtrB) and protein secretion (Type II, but not Types I and V) pathways involved in extracellular electron transfer to external Mn(IV) and Fe(III) oxides are also required for electron transfer to Mn(III). The inability of $\Delta omcA$ to reduce Mn(III) only with H₂ as electron donor suggests that OmcA is essential for H₂-dependent Mn(III) reduction. The inability of $\Delta mtrC$, $\Delta mtrA$, or $\Delta mtrB$ to reduce Mn(IV) oxide or Mn(III) regardless of electron donor suggests that the *S. oneidensis* extracellular electron conduit MtrCAB may reduce Mn(IV) oxide and Mn(III) via two successive one-electron reduction steps in a manner analogous to the two successive one-electron oxidation steps catalyzed by the purified Mn(II)/Mn(III) oxidase of *Bacillus sp.* strain PL-12 [47, 48]. As opposed to a single, two-electron transfer step, the two successive one-electron transfer steps of microbial Mn(IV) reduction are also predicted by voltammetric [83] and molecular orbital [55] analyses. The Mn(IV) oxide and Mn(III) reduction activities of OmcA and the MtrCAB complex are currently being examined after purification and reconstitution in proteoliposomes.

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CHAPTER 4

MICROBIAL MN(IV) REDUCTION IS INITIATED VIA A ONE-ELECTRON REDUCTIVE SOLUBLIZATION STEP

[The work described in Chapter 4 was done in collaboration with Dr. Lin Hui, a former PhD student in Dr. Martial Taillefert's research group. Dr. Lin's contributions in this chapter are denoted with an asterisk. The work described in Chapter 4 has also previously been published: Hui, L., N. Seinzbaum, T. DiChristina, M. Taillefert. 2012. Microbial Mn(IV) reduction requires an initial one-electron reductive solubilization step. *Geochim. Cosmochim. Acta*, 99:179-192].

Summary

Mn(IV) and Mn(II) are the most stable and prevalent forms of manganese in natural environments. The occurrence of Mn(III) in minerals and the detection of soluble Mn(III) in natural waters, however, suggest that Mn(III) is an intermediate in both the oxidation of Mn(II) and the reduction of Mn(IV). Here we show for the first time that microbial Mn(IV) reduction proceeds step-wise via two successive one-electron transfer reactions with production of soluble Mn(III) as transient intermediate. Only the second electron transfer step is coupled to production of dissolved inorganic carbon, suggesting that the first electron transfer reaction is a reductive solubilization step that increases Mn bioavailability. These findings oppose the long-standing paradigm that microbial Mn(IV) reduction proceeds via a single two-electron transfer reaction coupled to organic carbon oxidation, and suggest that diagenetic models should be revised to correctly account for the impact of manganese reduction in the global carbon cycle.

Introduction

Manganese is the third most abundant transition metal in the Earth's crust and plays an essential role in the biogeochemical cycling of carbon, nitrogen, phosphorus, and other metals [1-3]. Microbial Mn(IV) reduction is central to the biogeochemical cycling of many other metals and radionuclides in aquifers [4], redox stratified water columns [5], and fresh water [6] and marine sediments [7], yet the mechanism of dissimilatory Mn(IV) reduction by metal-reducing bacteria remains poorly understood. The ability to reduce Mn(IV) oxides is found in both domains of the prokaryotic world [8], including metal-reducing members of the genus *Shewanella* which reduce solid Mn(IV) [9, 10], soluble Mn(III) [11], and a wide variety of alternate electron acceptors, including solid and soluble forms of Fe(III) [12, 13].

Fe(III)- and Mn(IV)-reducing bacteria are presented with a unique physiological challenge at circumneutral pH: they are required to reduce electron acceptors found largely as amorphous or crystalline (oxy)hydroxide particles presumably unable to contact inner membrane (IM)-localized electron transport systems, where hydrogenases, menaquinone and ATPase are localized. To overcome this problem, metal-reducing bacteria employ novel electron transport strategies not found in other gram-negative bacteria that reduce soluble electron acceptors [6, 12], including 1) direct enzymatic reduction of solid metal oxides via metal reductases localized on the outer membrane (OM) or on electro-active appendages [14-17], 2) a two-step, solubilization and reduction

pathway in which solid metal oxides are first dissolved by organic complexing ligands, followed by uptake and reduction of the soluble organic-metal complexes by periplasmic metal reductases [18-20], and 3) a two-step, electron shuttling pathway in which exogenous or endogenous electron shuttling compounds are first enzymatically reduced and then chemically oxidized by the solid metal oxides in a second (abiotic) electron transfer reaction [21-23].

The mechanism of microbial Mn(IV) reduction is generally described as a single two-electron transfer reaction producing Mn(II) as end-product. In the present study, we demonstrate that the gram-negative, metal-reducing bacterium *S. oneidensis* MR-1 reduces Mn(IV) to Mn(II) step-wise via two successive one-electron transfer reactions with Mn(III) as transient intermediate, a hypothesis previously postulated on the basis of molecular orbital theory considerations [24]. The Mn(IV) reduction pathway of *S. oneidensis* therefore appears to be a reversal of the Mn(II) oxidation pathways of a *Bacillus* species and two recently isolated alphaproteobacteria which oxidize Mn(II) to Mn(IV) step-wise via two successive one-electron transfer reactions also with Mn(III) as transient intermediate [25, 26].

Materials and Methods

Strains used in this study

Shewanella oneidensis strain MR-1 was originally isolated from Oneida Lake, NY [9]. The *S. oneidensis* mutant strains used in the present study are listed in Table 2.1. All

strains were grown at 30°C in a defined salt medium (SM) supplemented with 18 mM lactate as carbon/energy source.

Preparation of amorphous Mn oxides

The amorphous Mn oxide was prepared by the oxidation of Mn^{2+} by potassium permanganate [27] (Equation 2.1), and modified to maintain sterile conditions [28].



Two separate 100 ml solutions, one with 0.30 M of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ and the other with 0.20 M of KMnO_4 , were prepared in volumetric flasks with sterile water. The Mn(II) solution was slowly dripped (≈ 2 drips/sec) the KMnO_4 solution to form black MnO_2 particles (250 mM). The protons produced during the reaction were neutralized by NaOH (10 N) to maintain the $\text{pH} \approx 10$ throughout the reaction. Prior to use in experiments, Mn oxides were washed in culture media to remove the excess Mn^{2+} .

Preparation of soluble Mn(III)-pyrophosphate

Mn(III) pyrophosphate stocks were made by adding Mn(III) acetate dihydrate (Sigma Aldrich, ACS grade) to sodium pyrophosphate (Sigma Aldrich, ACS grade) solution with a molar ratio of 1 Mn(III) : 4 pyrophosphate. The pH of the sodium pyrophosphate was adjusted to ≈ 8.2 before addition of Mn(III) acetate dehydrate and the final Mn(III) pyrophosphate stock was maintained at pH 8 [11] .

Anaerobic incubations of *S. oneidensis* wild-type and mutant strains with Mn(IV) oxide or Mn(III)-pyrophosphate as electron acceptor.

Duplicate liquid cultures were incubated anaerobically in 100 ml sealed reactors gently mixed with magnetic stir bars. Each incubation contained 1×10^7 cells/ml of *S.*

oneidensis wild-type or mutant strains incubated in 50 ml of SM medium with 18 mM lactate as electron donor and either 7 mM Mn(IV) oxide or 7 mM soluble Mn(III)-pyrophosphate as electron acceptor. Duplicate abiotic control incubations were conducted simultaneously with all biotic incubations.

Sampling and chemical analyses.

At each time point, a 2-ml aliquot was sampled with a sterile needle syringe from each batch reactor. 100 µl of unfiltered subsample was used to measure pH (MI-414 combination pH microelectrode, Microelectrodes, Inc. and SB301 SympHony pH meter, VWR Scientific). Another 1.5 ml subsample was filtered onto a 0.2 µm polyethersulfone membrane (Whatman) and divided into three aliquots to measure dissolved inorganic carbon (DIC), soluble Mn(II), and soluble Mn(III). The precipitate on the filter membrane was digested sequentially, first by a 0.5 M MgCl₂ solution to extract exchangeable Mn²⁺ ions adsorbed onto the solid surfaces, then by a 0.1 M NaOAc-HOAc solution (pH 5) for Mn species bound to carbonate [29]. Each extraction was conducted in duplicate. Mn(II) concentrations in all sequential extractions were measured by voltammetry and normalized to the volume of filtered solution to convert to concentration.

Total DIC was measured by a flow injection analysis system with conductivity detection [30] coupled to an Analytical Instruments System, Inc. (AIS, Inc.) LCC-100 integrator. Mn(II) concentrations in both the solution and solid extracts were determined by cathodic square wave voltammetry with a hanging mercury drop electrode (V663, Metrohm, Inc.) using an AIS, Inc. Model DLK-60 potentiostat. Square wave voltammetry parameters included a conditioning step for 10 sec at -0.1 V, a scan rate of 200 mV/s

from -0.1 to -1.8 V, and a pulse height of 0.05 V. Voltammograms were integrated using the semi-automatic integration program VOLTINT in Matlab [31]. Mn(III) concentrations were monitored spectrophotometrically at 480 nm (Milton Roy spectronic 501) in the presence of pyrophosphate [11]. Total Mn concentrations were measured by Graphite Furnace Atomic Absorption Spectrometry (GFAAS) with a pyrolytic-coated partitioned graphite tube (CPI international).

Results and discussion

The mechanism of microbial Mn(IV) reduction is generally described as a single two-electron transfer reaction producing Mn(II) as end-product [8]. In the present study, we demonstrate that the gram-negative, metal-reducing bacterium *S. oneidensis* MR-1 reduces Mn(IV) to Mn(II) step-wise via two successive one-electron transfer reactions with Mn(III) as transient intermediate, a hypothesis previously postulated on the basis of molecular orbital theory considerations [24]. Evidence for the two-step Mn(IV) reduction pathway of *S. oneidensis* is based on a comparison Mn(IV) and Mn(III) reduction and Mn(III) and Mn(II) production by *S. oneidensis* strains fed either Mn(IV) or Mn(III) as electron acceptor (Figure 4.1). The *S. oneidensis* strains include the wild-type strain (displaying normal Mn(IV) and Mn(III) reduction activities) and two sets of Mn reduction-deficient mutant strains (Table 1): the first is unable to reduce either Mn(IV) or Mn(III) as electron acceptor (*gspD* and *mtrB* gene deletion mutants, designated $\Delta gspD$ and $\Delta mtrB$, respectively), while the second is unable to reduce Mn(III), yet retains the ability to reduce Mn(IV) (Mn3-1 point mutant). *S. oneidensis* $\Delta gspD$ and $\Delta mtrB$ mutants are severely impaired in the ability to reduce Mn(IV) oxides, Fe(III) oxides [14, 32-34],

and Mn(III) (from results of the present study). GspD is an outer membrane channel-forming secretin required for extracellular transport of proteins, including decaheme *c*-type cytochromes MtrC and OmcA to the cell surface [15, 34], while *mtrB* encodes an outer membrane β -barrel protein that is postulated to anchor MtrC and OmcA on the cell surface and allows them to interact with MtrA during electron transfer to external Mn(IV) and Fe(III) oxides [35]. The Mn3-1 point mutant was recently identified in a genetic screen of chemically-mutagenized *S. oneidensis* strains selected for their inability to reduce Mn(III) (Chapter 2). Subsequent tests in anaerobic liquid culture demonstrated that the Mn3 point mutant was unable to reduce Mn(III), but reduced Mn(IV).

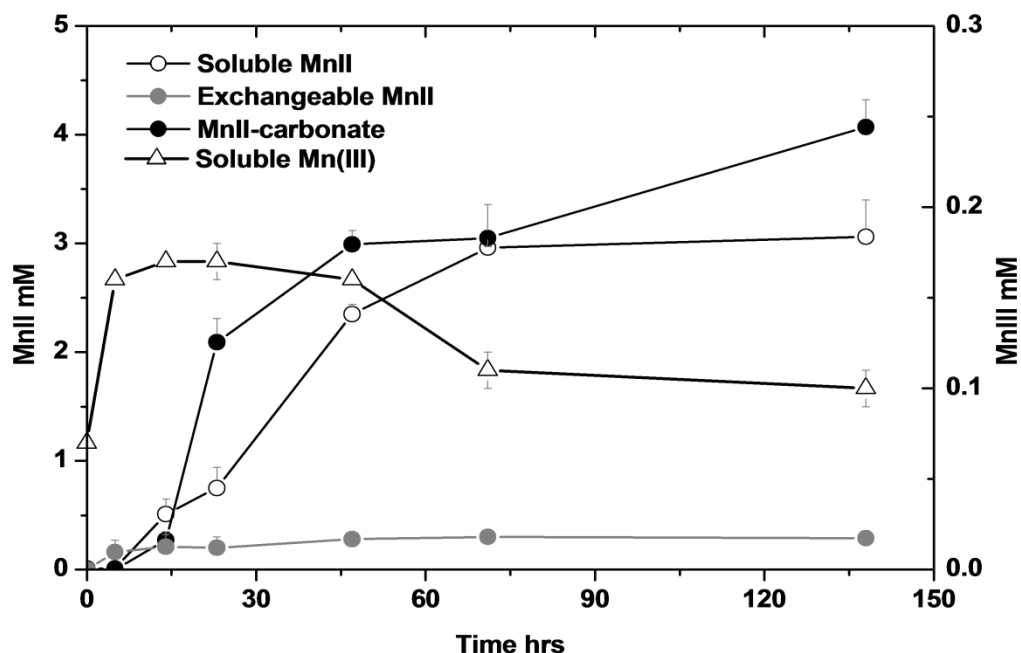


Figure 4.1. Production of Mn species during anaerobic respiration of solid Mn(IV) by *Shewanella oneidensis* MR-1. The Mn species detected include soluble Mn(II) (○), soluble Mn(III) (△), and sequentially exchangeable Mn(II) (●) and MnCO_{3(s)} (●).*

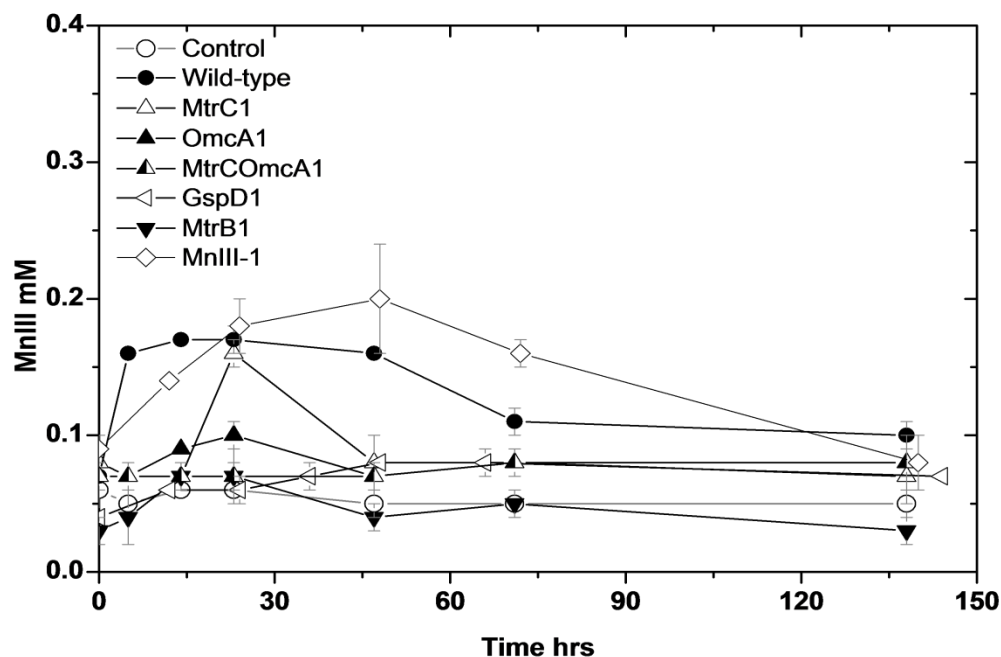


Figure 4.2. Production of soluble Mn(III) during anaerobic respiration of Mn(IV) by *S. oneidensis* wild-type and mutant strains. Wild type *S. oneidensis* MR-1 (●), $\Delta mtrC$ (△), $\Delta omcA$ (▲), $\Delta mtrC\text{-}omcA$ (◄), $\Delta gspD$ (◁), $\Delta mtrB$ (▼), Mn3 mutant (◇), and abiotic controls (○). (A) Soluble Mn(II) concentrations (mM) in different batch reactors. Error bars represent standard deviations from duplicate culture incubations.*

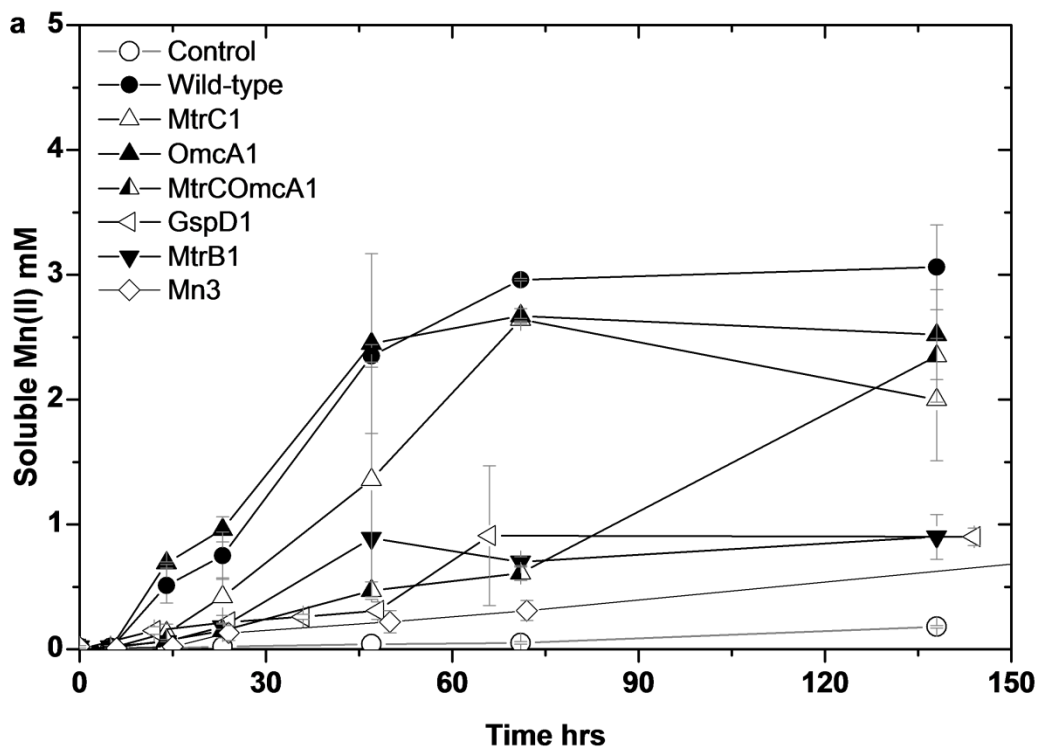


Figure 4.3. Production of soluble Mn(II) during anaerobic respiration of Mn(IV) by *S. oneidensis* wild-type and mutant strains. Concentration of Mn(II) produced during anaerobic respiration of solid Mn(IV) by wild type *S. oneidensis* MR-1 (●), $\Delta mtrC$ (△), $\Delta omcA$ (▲), $\Delta mtrC-omcA$ (▲), $\Delta gspD$ (◁), $\Delta mtrB$ (▼), Mn3 mutant (◇), and abiotic controls (○). (A) Soluble Mn(II) concentrations (mM) in different batch reactors. Error bars represent standard deviations from duplicate culture incubations.*

Production of both total Mn(II) and Mn(III) was significantly impaired during Mn(IV) oxide reduction by $\Delta gspD$ and $\Delta mtrB$ (Figures 4.2 and 4.3). These findings suggest that GspD and MtrB are both required to reduce Mn(IV) oxides, suggesting that Mn(IV) reduction shares components of the Fe(III) oxide reduction pathway [14, 32, 33]. Rates for Mn(IV) oxide reduction by the $\Delta omcA$, $\Delta mtrC$ or $\Delta omcA mtrC$ are approximately 99%, 70%, and 75% that of wild-type, (Figure 4.4), suggesting that, in the absence of MtrC and OmcA, alternative components of the metal reduction pathway are used during Mn(IV) reduction. To verify that Mn(IV) oxide reduction by *S. oneidensis* proceeds via two successive one-electron transfer reactions, Mn(III) reduction-deficient mutant Mn3-1 was tested for Mn(IV) reduction activity. Mn(III) reduction-deficient mutant strain Mn3-1 produced Mn(II) at levels only 17% of the wild-type strain, yet retained the ability to produce Mn(III) at wild-type levels when provided with Mn(IV) oxide as electron acceptor (Figure 4.4). The inability of Mn3-1 to reduce Mn(III) was verified in anaerobic liquid incubations with soluble Mn(III) as electron acceptor (Chapter 2). The point mutation in Mn(III) reduction-deficient mutant strain Mn3-1 is currently being identified via genetic and nucleotide sequence analyses.

Mn(III) reduction by *S. oneidensis* wild-type and mutant strains with Mn(III) as electron acceptor*.

The *S. oneidensis* wild-type and mutant strains were incubated on soluble Mn(III)-pyrophosphate complexes as a model soluble Mn(III) compound. Soluble

Mn(III) reduction activity is impaired in $\Delta mtrB$ and $\Delta gspD$ to $(20 \pm 4)\%$ and $44 (\pm 0.2)\%$ of wild-type activity, respectively; Figure 4.4). Involvement of MtrB and GspD in reduction of both solid Mn(IV) and soluble Mn(III) indicates that the reduction of both Mn(IV) and Mn(III) proceeds at the *S. oneidensis* OM. *S. oneidensis* most likely reduces soluble Mn(III) at the OM to avoid energetic costs associated with importing Mn(III) or exporting Mn(II) across the OM, or to avoid problems associated with intracellular Mn(II) toxicity after Mn(III) reduction [36].

$\Delta mtrC$ was severely impaired in Mn(III) reduction activity, while $\Delta omcA$ reduced Mn(III) at wild-type rates (Figure 4.4). This finding confirms that MtrC is required for Mn(III) reduction while OmcA is not required.

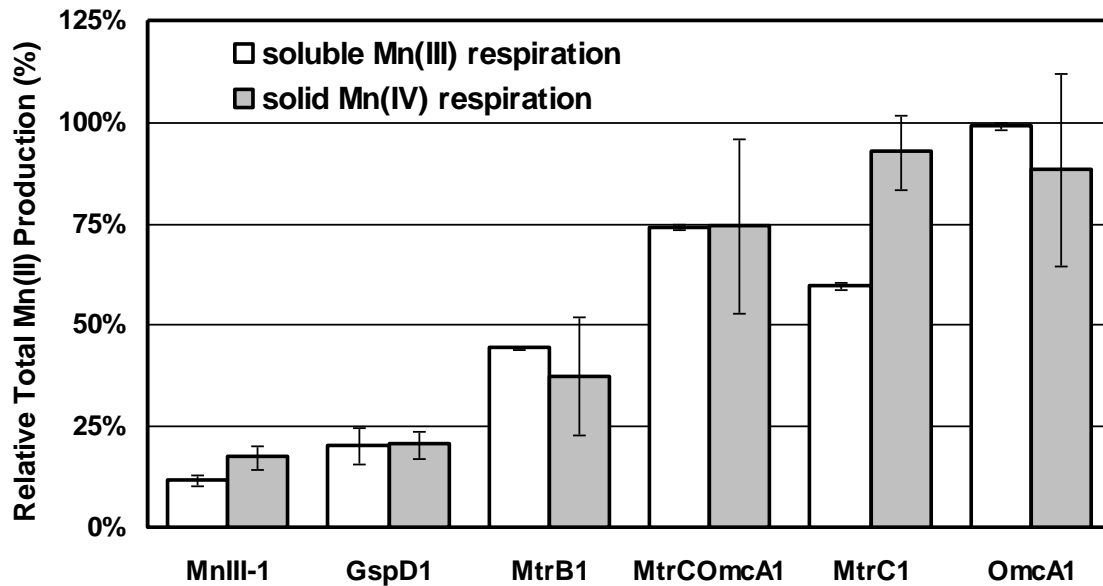


Figure 4.4. Total Mn(II) production at steady-state by different mutant strains of *S. oneidensis* MR-1. Values shown are relative to the wild type during incubations with either soluble Mn(III) (□) or solid Mn(IV) (■) as the terminal electron acceptor. *

DIC production by *S. oneidensis* wild-type and mutant strains fed Mn(IV) oxide or Mn(III) pyrophosphate as electron acceptor *.

Results of the present study indicate that Mn(IV) reduction by *S. oneidensis* proceeds via two successive one electron transfer reactions, with the possibility that either one or both are coupled to DIC production. Previous studies have reported that lactate, the carbon-energy source supplied in the anaerobic incubations, is oxidized to acetate and CO₂ by *S. oneidensis* during Mn(IV) oxide respiration [9]. Dissolved inorganic carbon (DIC) may therefore be used as a proxy for *S. oneidensis* lactate oxidation [37]. DIC concentrations increased to approximately 10 mM during anaerobic incubations of *S. oneidensis* on 18 mM lactate and either solid Mn(IV) oxides or soluble Mn(III) (Figure 4.5). The pH simultaneously increased by approximately 1.5 units (data not shown), reflecting lactate oxidation and proton consumption during Mn(IV) or Mn(III) reduction. DIC concentrations showed a positive correlation with total Mn(II) produced during the reduction of either solid Mn(IV) oxide or soluble Mn(III)-pyrophosphate as electron donor (Figure 4.5), indicating that Mn(II) production is tightly coupled to lactate oxidation as previously reported for Mn(III) reduction by *S. oneidensis* [11]. Assuming both electron-transfer steps are coupled to DIC production, Mn(IV) reduction is expected to produce twice as much CO₂ than that produced by Mn(III) reduction. However, similar concentrations of DIC were produced by *S. oneidensis* cultures incubated anaerobically with the same concentration of either Mn(IV) oxides or dissolved Mn(III) (Figure 4.5). These results indicate that *S. oneidensis* couples lactate oxidation with electron transfer to Mn(III) only, and not to reduction of Mn(IV). The electron transfer, therefore, seems to consist in a reductive solubilization step of Mn(IV)

to Mn(III) and an energy-generating step, of Mn(III) reduction to Mn(II). Recent studies on Fe(III) oxide reduction also proposed an initial solubilization step prior to the anaerobic reduction of Fe(III) oxides [18, 38]. While *Shewanella* may reduce Fe(III) oxides by non-reductively dissolving Fe(III) with exogenous or endogenous organic ligands, we propose that the paucity of ligands able to non-reductively solubilize Mn(IV) oxides at circumneutral pH in aquatic systems has forced *S. oneidensis* to evolve a Mn(IV) reductive solubilization-based strategy that produces soluble Mn(III) intermediates whose subsequent reduction is linked to organic carbon oxidation. The Mn(IV) reduction pathway of *S. oneidensis* appears to involve the OM *c*-type cytochrome MtrC and electron-shuttling compounds [39]. These findings have important implications for our understanding of the biogeochemical cycling of Mn in aquatic systems. First, Mn(III) production during Mn(IV) oxide reduction may explain the existence of soluble Mn(III) in suboxic or anoxic water columns and sediments [40]. Second, Mn(IV) oxide is a readily available terminal electron acceptor for the mineralization of organic compounds in anaerobic environments [7, 41]. If the first electron transfer reaction proceeds via a reductive solubilization step to activate Mn while the second step is coupled to CO₂ production, then diagenetic models which estimate the contribution of manganese reduction to carbon remineralization based on the transfer of two electrons [1, 42, 43] should be revised to more accurately quantify the global carbon cycle.

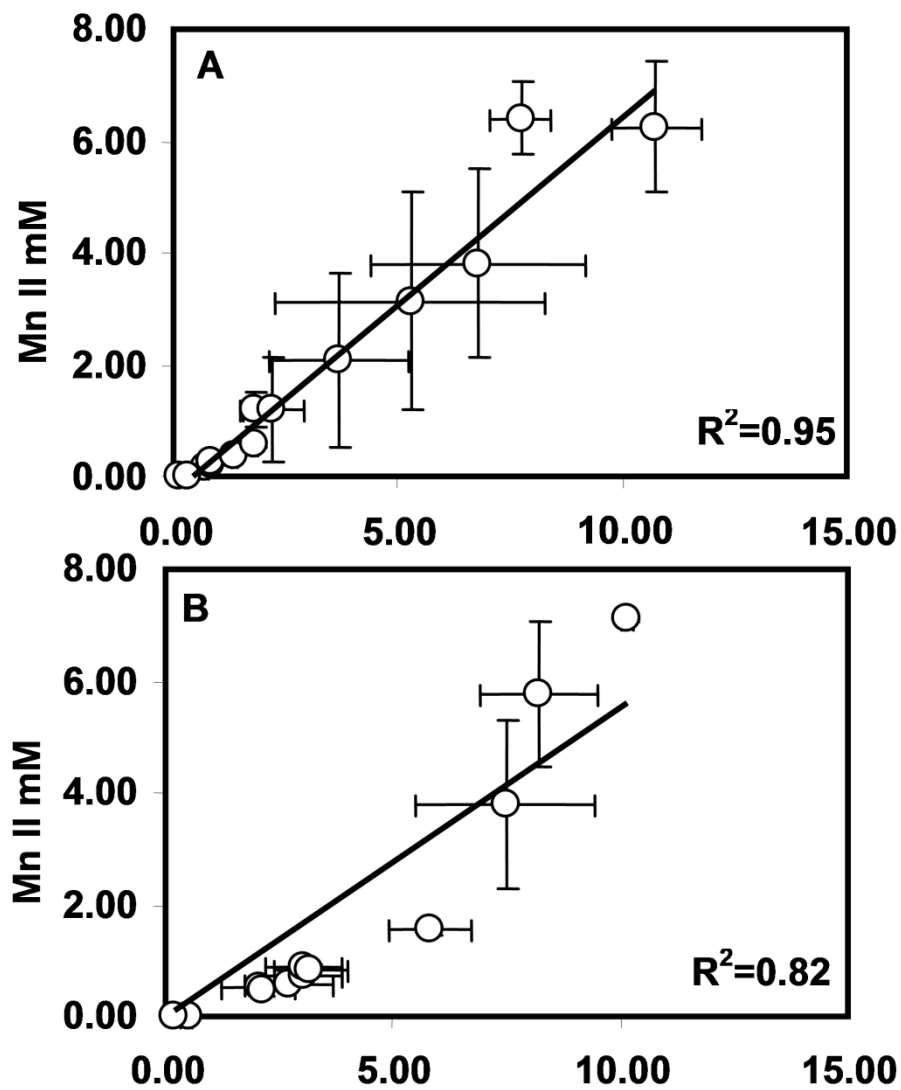


Figure 4.5 Correlation between DIC production and Mn(II) production during A) Mn(IV) or B) Mn(III) reduction.*

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CHAPTER 5
ANAEROBIC MICROBIAL CONSORTIA
CONSISTING OF EXOELECTROGENIC
***OCHROBACTRUM* AND METAL-**
REDUCING *SHEWANELLA SPP.* ARE
SELECTED DURING ENRICHMENT
UNDER NH_4^+ -OXIDIZING AND Mn(III)-
REDUCING CONDITIONS

[The work described in Chapter 5 was done in collaboration with Dr. Lin Hui, a former PhD student in Dr. Martial Tallefert's research group. Dr. Lin's contributions in this chapter are denoted with an asterisk].

Summary

Recent studies have shown that soluble Mn(III) can be one of the most abundant soluble Mn species in natural environments, but knowledge about microorganisms responsible for Mn(III) reduction in nature is scarce. Sediment profiles suggest that Mn reduction coupled to NH_4^+ oxidation is occurring within the upper 5 cm of salt marsh sediments (Skidaway, GA). The main objective of the present study was to enrich for microbial populations involved in anaerobic Mn(III) reduction coupled to ammonium oxidation in the absence of an added organic carbon source. Anaerobic batch cultures were set up with NH_4^+ as sole electron donor and Mn(III) as sole electron acceptor, and tested for Mn(III) reduction activity. After a 2-year enrichment process in batch liquid cultures, the resulting enriched microbial community displayed the ability to deplete NH_4^+ and produce Mn(II). Populations of the genus *Shewanella* and *Ochrobactrum* spp. dominated the

community at approximately 90% and 10% abundances, respectively. Two isolates strain TD-01 and TD-02, closely related to *S. haliotis* and *Ochrobactrum spp.*, respectively, were recovered from the enrichment, and tested for Mn(III) and Fe(III) reduction activity. Strain TD-01 reduced Mn(III) and Fe(III). The role of *Ochrobactrum*, however, is unknown. This is the first report of metal reduction ability in *S. haliotis*. In addition, TD-01 is the first reported *Shewanella* strain able to use acetate as an electron donor during metal reduction. Acetate utilization during metal reduction by strain TD-01 is unprecedented in the *Shewanella* genus and raises questions regarding the evolution of functional diversity of the *Shewanella* genus. This study expands the metabolic capacity of the genus *Shewanella*.

Introduction

Manganese (Mn) is an essential nutrient present in all living organisms [3]. Microorganisms can accelerate Mn redox transformation by orders of magnitude compared to abiotic reactions [4]. In the environment, Mn interacts with many biologically relevant elements. Mn oxides, for example, are strong oxidants of organic matter [5-7], inorganic compounds [8-10] and strong adsorbants of phosphorous. Mn can also adsorb metals and radionuclides [11]. Mn-reducing bacteria, therefore, play an important role in a variety of important environmental cycles. Heterotrophic Mn(IV)

respiration can be the dominant process for organic carbon oxidation in manganese-rich sediments [12, 13]. Mn(IV)-reducing bacteria can also utilize hydrogen (H₂) as electron donor [14]. To date, however, no organisms have been found that are capable of Mn reduction using an inorganic electron donor other than H₂. Recent voltammetric analyses have shown that 85-100% of the total soluble Mn pool in redox-stratified marine water corresponds to soluble Mn(III) chelated to organic ligands [15, 16]. Mn(III) may originate during oxidation or reduction of Mn and persist as Mn chelates by ligands of biological [17, 18] or synthetic origin [19]. The only account of biological Mn(III) reduction is Mn(III)-pyrophosphate reduction by *Shewanella oneidensis* MR-1. *Shewanella* uses the extracellular Fe(III) and Mn(IV)-reducing Mtr pathway for Mn(III) reduction, suggesting that Mn(III) is reduced extracellularly. While microbial Mn(III) redox transformations may represent a major redox process in marine and freshwater systems, little is known about the identity of the Mn(III)-reducing microbial populations.

NH₄⁺ is a limiting nutrient in many natural aquatic environments [20]. The pool of NH₄⁺ in aquatic environments originates from microbial N₂ fixation. Fixed nitrogen in the biosphere corresponds to less than 0.1% of the N pool and limits primary production in both terrestrial and marine ecosystems [20, 21]. Microbial Mn transformations may also drive the N cycle [22, 23]. The knowledge about NH₄⁺ fluxes by ammonium-oxidizing microorganisms, however, is still incomplete [24]. The anaerobic oxidation of ammonia (Anammox pathway) couples the oxidation of NH₄⁺ to NO₂⁻ reduction to produce N₂ via hydrazine by Planctomycetes [25]. Ammonia oxidation under anoxic conditions was first observed in wastewaters [26], and later studies showed that Anammox is a dominant process in many natural aquatic systems [27-30], responsible for

up to 50% of NH_4^+ mineralization [24, 31]. Previous studies have proposed that a biochemical pathway involving NH_4^+ as electron donor and Mn(IV) as anaerobic electron acceptor is thermodynamically favorable [32]. Sediment depth profile measurements at various locations revealed Mn(II) production profiles that overlap with NH_4^+ depletion profiles [33], or NO_3^- accumulation [30, 34] which suggests that anaerobic NH_4^+ oxidation coupled to Mn(IV) oxide reduction may occur in those sediments [33, 35]. In addition, slurry incubations studies have shown a positive correlation between ammonia oxidation (or disappearance) and Fe(III) oxide [36] or Mn(IV) oxide reduction [35]. Other studies have shown that anaerobic methane oxidation is associated with Mn(IV) and Fe(III) oxide reduction in slurry incubations [37] and with O_2 reduction from the product of NO_2^- disproportionation by members of an enrichment culture [38]. Methane-oxidizing bacteria also possess the ability to transform ammonia owing to the broad substrate specificity of the particulate methane monooxygenase enzyme (pMMO) [39]. Identifying organisms responsible for dissimilatory metal reduction in the environment is complicated because undefined media such as slurries, sludges, and sediments are often too complex to determine the metabolized redox substrates. In addition, metal reduction occurs outside the cell, and assimilation-based detection methods such as stable isotope probing (SIP) cannot be employed. Enrichment of natural communities with the capability to utilize the desired electron couple is therefore the only way to provide information about the existence of DMRB present in the environment.

The objective of this work was to test the hypothesis that bacteria can utilize NH_4^+ as electron donor and Mn(III) as an electron acceptor in a defined growth medium. To achieve this objective, a microbial enrichment strategy for growth on NH_4^+ and Mn(III)

was designed without the amendment of organic carbon sources to avoid selecting for metal-reducing heterotrophs. After a 2-year sequence of transfers, the Mn(III) reducing culture was 90% enriched in *Shewanella* spp. and 10% *Ochrobactrum* spp. The enriched culture did not contain added organic carbon and thus reduced Mn(III) with NH_4^+ as sole electron donor.

Materials and methods

Sampling site*

Sediments used as initial inoculum were collected in March 2010 from a perennial creek bank site (CB) at the salt-marsh Ecosystem Research Facility (SERF) of Skidaway Institute of Oceanography, Georgia (Figure 5.1) [40]. Iron and manganese respiration is the primary microbial process for carbon re-mineralization at these locations of the marsh, where suboxic conditions dominate, maintained by high hydrostatic pressure gradients during tidal cycles [41]. Triplicate sediment cores were collected using a corer (50 cm long and 7.5 cm diameter) with a long handle to sample submerged sediments without disturbing the nearby sediment.

Vertical pore-water profiles of the main redox species involved in diagenesis were obtained voltammetrically. Sediment core profiles revealed a region between the upper 3-5 cm with overlapping Mn(II) peaks, depleted NH_4^+ , Fe and HS^- , suggesting that anaerobic ammonia oxidation coupled to Mn(IV) reduction was the prevalent microbial process at that depth interval

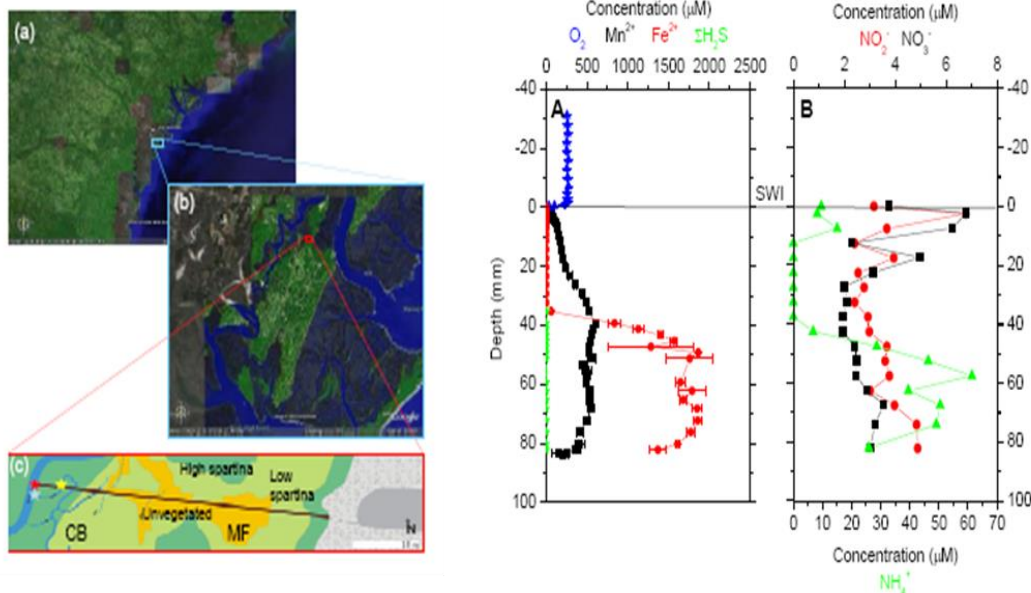


Figure 5.1*. Sampling site and representative depth profile of substrates and products involved in microbial anaerobic respiration [40].

Culture media and growth conditions

Enrichment strategy

Sediments (0.025 g/mL) were transferred into Hungate tubes under 85% N₂, 10% H₂, and 5% CO₂ in an anaerobic chamber. The bottles were sealed with thick butyl rubber stoppers and the headspace was flushed with N₂ gas and incubated in the dark at room temperature. Samples were transferred with an N₂-flushed syringe into bottles under anaerobic conditions by flushing the headspace with N₂ gas for 30 minutes. After three serial transfers in 20 mL Hungate tubes, cultures were incubated in 160 mL serum bottles. Each bottle contained 100 mL of nitrogen- and sulfur-free artificial seawater [42] (Table 5.1) diluted to represent salt marsh salt concentrations. Appropriate concentrations

of NH_4^+ and SO_4^{2-} , nutrients, and inhibitors were added depending on the required experimental conditions. A vitamin mix solution (Table 5.2) was added to all incubations, except for no vitamin controls where indicated. Vitamin concentrations are listed in Table 5.2. Anaerobic Mn(III) was added with a syringe after the bottles were made anaerobic. Oxygen was not detected in the samples (oxygen detection limit: 250 ppb) and periodic controls of rezazurin in sacrificial bottles confirmed anaerobic conditions.

Mn(III) reduction as a function of sulfate concentration

Enriched cultures were amended with 100 μM , 1 mM, or 14 mM sulfate with either 70 μM or 1 mM NH_4^+ to determine if the concentration of sulfate affected the rate of Mn(III) reduction. Mn(III)-pyrophosphate was added at 200 μM .

Mn(III) reduction as a function of sulfate and organic sulfur additions

The enriched cultures were amended with SO_4^{2-} (10 μM and 14 mM concentrations), cysteine, methionine or cystine (10 μM each). All bottles contained NH_4^+ (1 mM). Mn(III) was added at 200 μM . Controls included SO_4^{2-} at 10 μM , and organic carbon controls for each organic carbon treatment as serine (cysteine and cystine control) and valine (methionine control), with 10 μM SO_4^{2-} as sulfur source for growth. Bottles were inoculated at 5% final concentration from the third generation culture.

Respiratory and growth inhibitor controls

Enriched cultures incubated with SO_4^{2-} (14 mM), NH_4^+ (1 mM), and Mn(III) (200 μM) were amended with the sulfate reduction inhibitor molybdate (14, 28, 70 and 140 mM), ampicillin (1.1 and 2.3 mM), chloramphenicol (61.9 and 619 mM), 2,4 dinitrophenol (360 and 3600 μM), mercuric chloride (1mM) and Carbonyl Cyanide m-

Chlorophenyl Hydrazone (CCCP, 200 μ M). Bottles were inoculated at 5% final concentration from the third generation culture.

Isolation of colonies that reduce Mn(III)

Microorganisms were isolated from the enrichment cultures in rich media plates (LB-ASW agar). Single colonies were grown in ASW with NH_4^+ and Mn(III) and reintroduced into Mn(III)-pyrophosphate amended serum bottles to determine Mn(III) reduction ability, and serial dilutions were re-grown in rich media again to ensure that only one colony type was obtained.

Table 5.1. Artificial seawater composition

	g/L
NaCl	30
MgCl	1
Na ₂ SO ₄	4
KCl	0.7
CaCl ₂	0.15
NaHCO ₃	0.2
MOPS	2.09
KH ₂ PO ₄	0.27
KBr	0.1
SrCl ₂	0.04
H ₃ BO ₃	0.025
KF	0.001

Table 5.2. Vitamin mix composition. Final concentration in seawater and nutritional properties

Vitamin	μM	per molecule				
		S	N	C	amino groups	acid groups
p-aminobenzoic acid	1.46		1	7		1
Folic acid	0.23		7	19		2
Thioctic acid	0.48	2		8		1
Riboflavin	0.53		4	17		
Thiamine	1.33	1	4	12	1	
pPanthothenic acid	0.91		1	9		1
Cobalamin	0.15		14	63	5	
Biotin	0.16	1	2	10		1
total (μM)	5.25	2.46	13.80	70.28	2.91	2.91

Chemicals

Amorphous Mn(IV) oxide and Mn(III)-pyrophosphate stocks were prepared as described in the previous chapters. Acetate-free Mn(III)-pyrophosphate was prepared by solubilization of 10 mM Mn(III)-acetate or 500 mM Mn_2O_3 (acetate-free Mn(III)) in 40 mM sodium pyrophosphate at pH 8 and 6.5, respectively [19].

Analytical methods

NH_4^+ was measured by the phenol hypochlorite method [35]. Samples were oxidized by a 10% phenol solution in water, and reacted with a 1% alkaline hypochlorite

solution catalyzed by sodium nitroprusside. NO_2^- was measured spectrophotometrically with sulfanilic acid-*N*-1-naphthyl-ethylene-diamine dihydrochloride solution. Mn(II) and HS^- concentrations in both the solutions and solid extracts were determined by cathodic square wave voltammetry with a hanging mercury drop electrode (HMDE) using an AIS, Inc. Model DLK-60 potentiationstat. Square wave voltammetry parameters included a conditioning step for 10 sec at -0.1 eV, a scan rate of 200 mV/s from -0.1 to -1.8 V, and a pulse height of 0.05 V. The voltammograms were integrated using the semi-automatic integration program VOLTINT in Matlab [43]. Photographic images were obtained with a Canon model PowerShot A620 of 7.1 megapixels.

DNA isolation

DNA was obtained from biomass of culture collected by centrifugation (10,000 $\times g$, 25 min at RT), of a 5th generation batch-grown culture at day 60, which had reduced 200 μM Mn(III). Cell lysis was achieved by incubation for one hour at 37°C in 1% SDS and 10 $\mu\text{g/L}$ lysozyme (Sigma) followed by addition of proteinase K (60 mg/ml) (Sigma) and 5U of RNaseA (Sigma), and incubation for 1 hour at 37°C. DNA was extracted and purified with phenol:chloroform:isoamyl (25:24:1) followed by chloroform:isoamyl (24:1) [44]. DNA was precipitated with 0.1 volumes of sodium acetate 3 M pH 5.5 and 0.7 volumes of isopropanol, washed with 70% ethanol, and finally resuspended in 50 μL of sterile water. The concentration of DNA was quantified by nanodrop at an absorbance of 260 nm.

Phylogenetic Analysis of Sequence Data

16 rDNA was amplified using universal primers U1 corresponding to the hypervariable 16S DNA gene region V1-3 to obtain amplicons of ≈ 200 pb (Table 5.1). Approximately 30 ng of DNA was used in a 50- μ l PCR reaction mixture. One unit of iProof DNA polymerase (BioRad) was used in the PCR reaction mixture with an annealing temperature of 40°C in a buffer supplied by the manufacturer. Duplicate PCR products were pooled to minimize possible bias due to random events within individual PCR reactions. The PCR bands were purified (Qiagen gel purification kit) prior to library construction. Amplified products were sequenced via ion torrent technology. The sequences obtained were analyzed using the open source, web-based platform for genomic analyses Galaxy [45, 46]. The workflow consisted of converting FastQ files to FASTA which were subsequently filtered by length to include only those larger than 80 pb in further analyses. Alignments were fetched by the software from the NCBI database (as of May 2012) and percentages of the genus obtained were calculated in Excel.

Quantitative PCR

qPCR was done in MicroAmp™ Fast Optical 96-Well Reaction Plate sealed with an Optical Adhesive Cover (ABI) using 2X Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) and an ABI 7500 Fast Real-Time PCR System equipped with SDS v. 2.0.3 software using the default SYBR Green cycling parameters qPCR master mix (ABI) with singleplex fluorescence detection. Eubacterial qPCR reactions were carried out with the following thermal program: 50°C for 2 min (1 cycle);

95°C for 10 min (1cycle); 95°C for 15 sec, 60°C for 1 min (40 cycles). Fluorescence data were collected at 60°C during each cycle. Calibration curves were obtained using 10-fold serial dilutions of plasmids carrying a single, cloned *Dehalococcoides* 16S rDNA gene using the Invitrogen TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) [1]. A calibration series was used for each qPCR reaction plate, and copy numbers of unknown samples were calculated. Samples and standards were run in triplicate qPCR reactions. The quantification of 16S rRNA gene copies was utilized as an approximation of cell numbers.

Phylogenetic identification of isolates

Single colonies were grown in ASW-LB and total DNA was extracted by the phenol:chloroform method described above and used for PCR amplification of full-length 16S DNA. Primers used were 8F and 1489R (Table 5.1) with 60°C annealing temperature. Amplicons were sequenced on both ends and joined to create a nearly complete 16rRNA gene sequence. Each nearly full-length sequence was identified by an nBLAST search (NCBI) using the nucleotide collection and megablast option.

Table 5.3 Primers used in this study

Name	Sequence	Description	Expected product length (bp)	Use	Ref
U1F	GGATTAGATACCCBGG	Universal – V1-3 hypervariable region	≈200	ion torrent sequencing	
U1R	CCCCGRCAATTCKTTYAGT				
1050	ACGGGCGGTGTGTAC	Universal primer	≈350	qPCR	[1]
1392	ATGGYTGTCGTCAGCT				
8F	AGAGTTTGATCCTGGCTCAG	Universal primer	≈1480	amplification 16S DNA	[2]
1489R	TACCTTGTTACGACTTCA				

Results and Discussion

Enrichment strategy: Mn(III) reduction with NH_4^+ as sole electron donor

Microbial Mn(III) reduction may be an important yet overlooked terminal electron-accepting process in aquatic environments [47], although the only known bacterial species able to reduce Mn(III) is *S. oneidensis* MR-1. *S. oneidensis* is able to reduce Mn(III)-pyrophosphate in minimal or rich growth media with lactate [35], formate, or hydrogen (Chapter 3) as electron donor. Other organisms able to reduce Mn(III), however, have not been identified. In the present study, an enrichment strategy was carried out in defined artificial seawater with Mn(III) as electron acceptor and NH_4^+ as electron donor without fixed carbon sources other than inorganic carbon as NaH_2CO_3 .

Production of Mn(II) was detected voltammetrically in 200 μM Mn(III)-amended microcosms. At least six generations of 5% v/v transfers of an enrichment culture showed Mn(III) reduction to Mn(II). Mn(II) was not detected in the abiotic controls or unamended microcosms.

Effect of SO_4^{2-} concentration on Mn(III) reduction

Previous authors have observed NH_4^+ oxidation coupled to sulfate reduction [48, 49]. In addition, sulfides can reduce Mn(III) abiotically [9]. Although sulfate was added to the media at 15 mM, corresponding to concentrations found in salt marsh waters, sulfide was not detected before nor immediately after Mn(II) was produced, nor in incubations with Mn(III) omitted (sulfate reduction controls). Further tests were carried out to determine whether sulfate was involved in Mn(III) reduction. The sixth generation enrichment culture was used

as inoculum for incubations with varied concentrations of SO_4^- to ensure that sulfide, the product of SO_4^{2-} reduction, was not acting as an abiotic Mn(III) reductant. Mn(II) production was identical in rates and extent under SO_4^{2-} concentrations of 100 μM , 1 mM, and 14 mM (Figure 5.1). Sulfide was not detected at any point throughout the experiment. The effect of molybdate, an inhibitor of sulfate reduction, on Mn(III) reduction was determined by monitoring Mn(III) spectrophotometrically using Benzidine because molybdate inhibits the functioning of mercury electrodes and it was not possible to measure soluble Mn(II) via voltammetry in these incubations. Molybdate addition did not inhibit Mn(III) reduction, as compared to unamended controls (Figure 5.4).

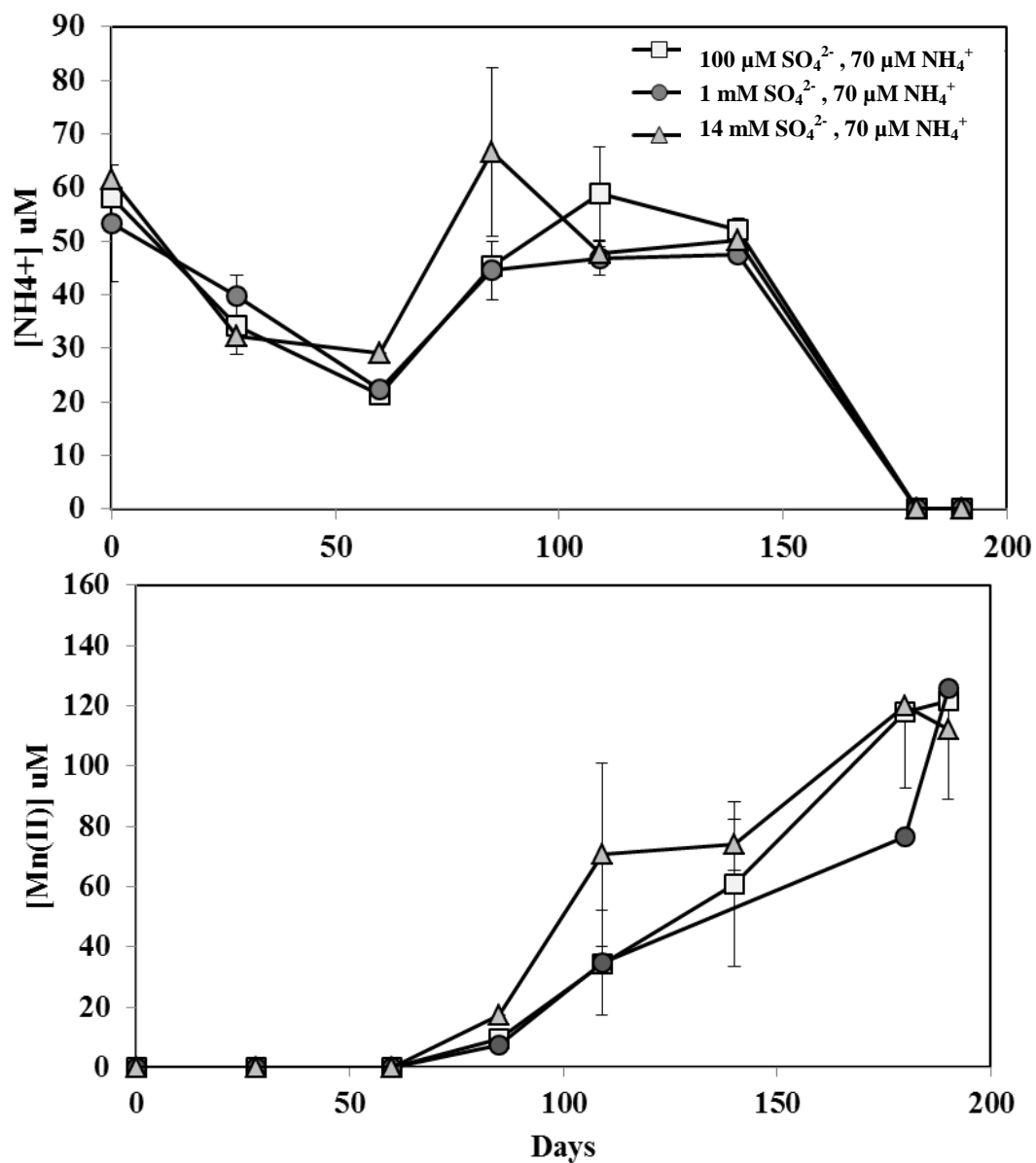


Fig 5.2. Rates of (A) Mn(III) reduction and (B) NH₄⁺ consumption over time of the enrichment culture incubated under anaerobic conditions with different SO₄²⁻ concentrations.

Interestingly, NH_4^+ levels displayed an initial drop of about 30 μM during the first 60 days, which rose again to initial values between days 50-80 and remained stable during Mn(II) production and then decreased to zero after Mn(III) was depleted at 150 days in all the incubations. This difference could be the result of coexisting populations of *Ochrobactrum* sp. (see below) that assimilated NH_4^+ in the enrichment culture initially before being overtaken by the *Shewanella* population that depleted Mn(III). Quantitative determination of copy numbers of core genes such as 16S rDNA genes specific to each population should determine whether this hypothesis is valid.

During all the incubations, the total Mn^{2+} concentration measured never added up to the initial 200 μM of Mn(III) added to each culture. During Mn(III) reduction, a white precipitate formed, possibly corresponding to Mn(II) carbonate or phosphates. The precipitates, undetectable with voltammetry, may account for the observed Mn(II) mass balance deficiency [50].

Effect of organic sulfur concentration on Mn(III) reduction

Microcosms were amended with organic sulfur sources to test the hypothesis that organic sulfur acts as an electron shuttle for manganese reduction. Two sets of amino acids were added: cystine, cysteine and methionine were added as sulfur sources; and serine and valine (which have the same number of C as the former group but lack S) were added as carbon source controls. Carbon source controls were also amended with 10 μM SO_4^{2-} as a S nutrient supplement. After 200 days of incubation, Mn(II) was produced in all of the treatments but not in any of the abiotic controls. SO_4^{2-} -amended enrichments

including the carbon source controls showed the fastest appearance of Mn(II) production.. These controls were followed in order of Mn(II) appearance by the enrichments amended with cysteine, then methionine, and finally cystine. The control incubation with SO_4^{2-} omitted also displayed Mn(II) production, although the starting point was 60 days, potentially after background levels of SO_4^{2-} carried over from the previous incubations were reduced. Interestingly, organic carbon amendment did not result in faster Mn(III) reduction rates. These results demonstrate that organic sulfur amendments do not result in increased Mn(III) reduction rates and are therefore most likely not involved as electron shuttling redox mediators, suggesting that the enriched phenotype involves a direct Mn(III) reduction mechanism.

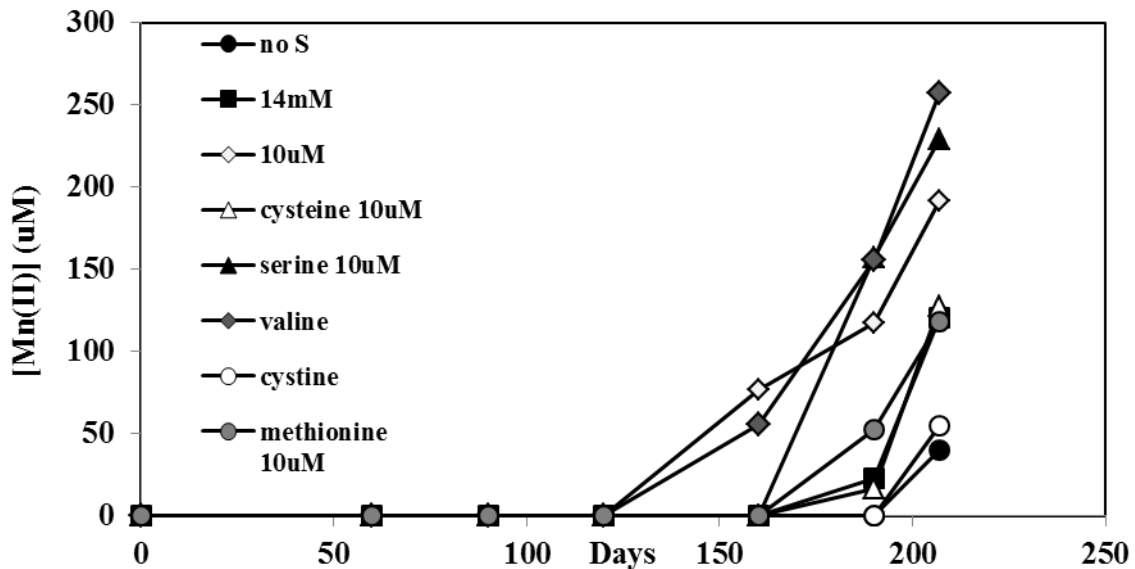


Fig 5.3. Rates of Mn(II) production during anaerobic incubation of the enrichment culture with Mn(III) as electron acceptor and amended with various organic sulfur sources and their carbon controls.

Respiratory inhibitors effect on Mn(III) reduction

Molybdate (10 mM), ampicillin (2.3 mM), and lower concentrations of dinitrophenol (360 μ M) and chloramphenicol (61 μ M) did not interfere with Mn(III) reduction. Chloramphenicol (620 μ M) inhibited Mn(III) reduction by 70%; DNP (3.6 mM) by 95%; CCCP (200 μ M) by 90%; and a SO_4^{2-} -omitted control microcosm by 85% (Figure 5.4). The inhibitory effect of antibiotics (chloramphenicol) and respiratory inhibitors (DNP and CCCP) demonstrate that Mn(III) reduction is a respiratory process.

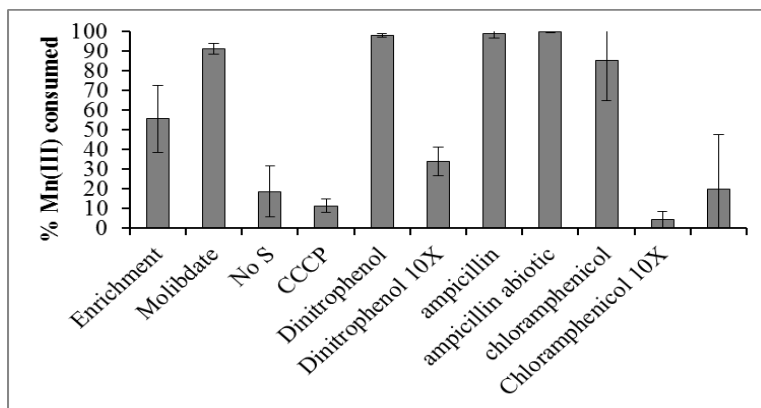


Figure 5.4. Extent of Mn(III) reduction of the enrichment culture incubated for 60 days under anaerobic conditions and amended with various respiration or growth inhibitors. The results are the means of duplicate determinations and standard deviations are shown as error bars.

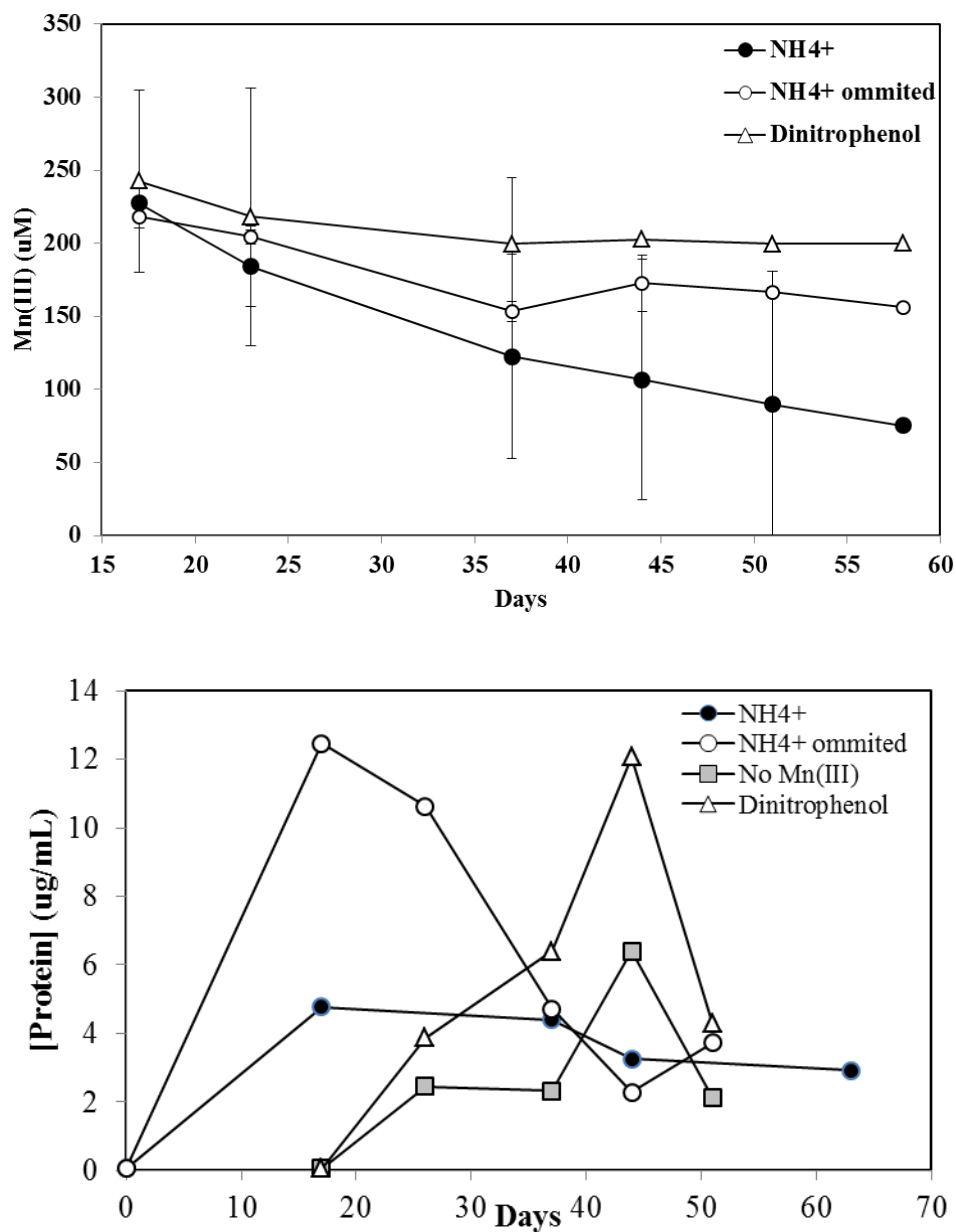


Figure 5.5. Mn(III) reduction and protein production by the enrichment culture during growth with NH₄⁺ as electron donor and Mn(III) as electron acceptor. A) Mn(III) reduction and B) protein levels of the culture and controls.

Quantitative PCR with universal primers for the 16S rDNA gene (Table 5.3) was used as a measure of cell density after 60 days of incubation, when all Mn(III) was reduced to Mn(II). Serial dilutions of genomic DNA amplified along the calibration standard curve (108%, slope -3.16), indicating that no contaminating PCR inhibitors were present that might result in PCR bias. The total 16S rDNA gene copy number increased by three orders of magnitude from 1.00×10^5 to $1.99 \times 10^8 \pm 7.54\text{E}+07$ per mL in 60 days of incubation, demonstrating that an increase in cell density was associated with Mn(III) reduction.

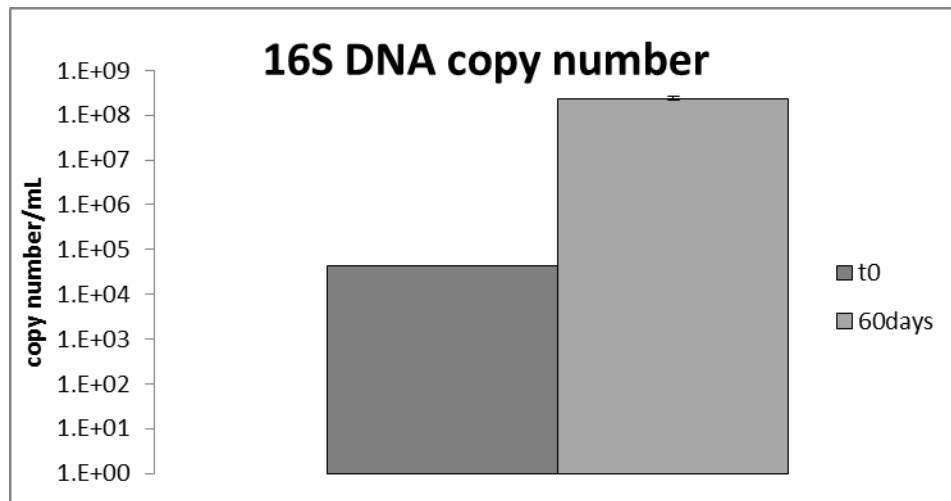


Figure 5.6. 16S rDNA gene copy number of the enrichment culture grown for 60 days under anaerobic conditions with NH_4^+ as electron donor and Mn(III) as electron acceptor. The results are the means of triplicate determinations, and standard deviations are shown as error bars. Some error bars are too small to be observed.

Identification of dominant populations in the enriched culture

16S rDNA gene amplification with universal primers and sequencing showed that 90% of the sequenced reads corresponded to members of the *Shewanella* genus, followed by less than 10% of *Ochrobactrum* species followed in abundance by *Marinobacter* (Figure 5.7).

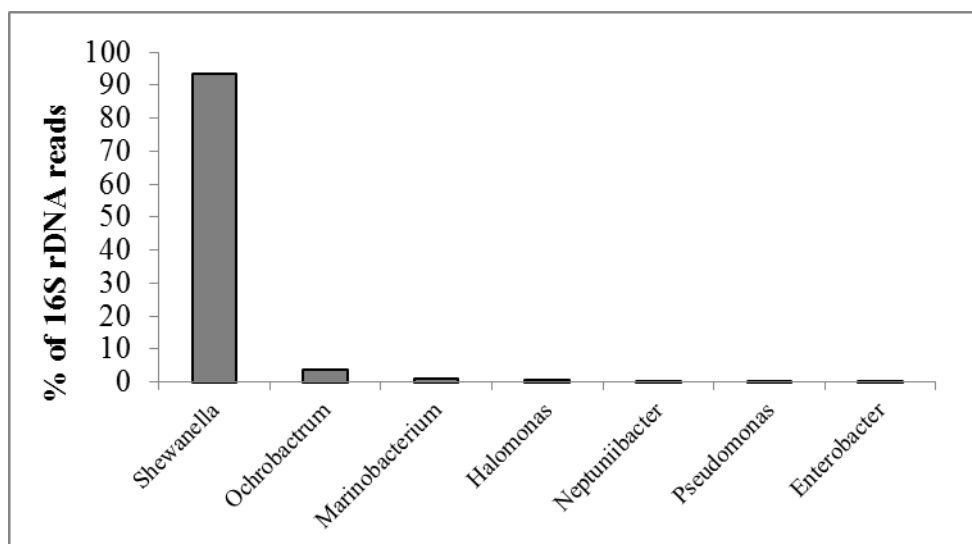


Figure. 5.7. Abundance of populations in the enriched culture after Mn(III) reduction based on number of reads from community 16S rDNA sequencing.

Marinobacter includes 21 species that have been isolated from the deep ocean [38, 51] and can also be found in a wide range of marine environments [52].

Marinobacter species often grow by denitrification, can resist Mn(II) concentrations of up to 10 mM and may oxidize Mn(II) [15, 16, 19]. Coincidentally, *Marinobacter* strains were also isolated from an Mn(IV)-containing anaerobic enrichment with succinate, lactate, and pyruvate as carbon sources, and possibly other electron donors [35]. Bacterial

species often follow an opportunitroph lifestyle [53]. Opportunitrophs have been recently defined as “organism(s) equipped to take advantage of transient occurrences of high-nutrient niches within a bulk low-nutrient environment....As a result, opportunitrophs are not generally observed to dominate a particular niche but rather employ a strategy of survival under any circumstance”. *Marinobacter* may be an example of an opportunitroph [54]. A review of isolation strategies for *Shewanella* revealed that *Shewanella* strains are easily cultured when enrichment from an environmental sample is followed by growth in nutrient rich media [19, 22]. *Shewanella* strains appear to have a selective advantage in low nutrient environments and are able to grow quickly in nutrient rich media. The *Shewanella* strain in the present study was enriched in minimal media with 200 μM Mn(III) as sole electron acceptor and NH_4^+ as sole electron donor, and requires approximately 60 days to reduce Mn(III) but is able to reduce Mn(III) using lactate as electron donor in less than 2 days. These characteristics may indicate that members of the *Shewanella* genus are also opportunitrophs. As typical *Shewanella* genome sizes are in the 5 Mbp range, this finding indicates that only 10% are shared, and 90% are strain-specific. Genomic analysis that found 40% of shared genes among 10 different *Shewanella* strains [55] may explain why *Shewanella* strains are so ubiquitous and able to survive environmental conditions that fluctuate between extreme starvation and high nutrient load. An *Ochrobactrum* species has been recently isolated from an enrichment culture that selected for organisms capable of extracellular electron transfer [56]. This species, however, was unable to reduce metals. *Ochrobactrum* have also been isolated from soil as well as from clinical samples

Isolation and identification of dominant populations

Two isolates were selected for further study as they were the most abundant populations as shown by the community 16S rDNA analysis described above. Single colonies were transferred to fresh rich media (LB-ASW) plates until they appeared pure. Nearly complete 16S rDNA gene sequences revealed that TD-01 is a member of the *Shewanella* genus and displayed 100% identity with 16S rDNA sequences from *S. haliotis*. Strain TD-02 belongs to the genus *Ochrobactrum* (Table 5.4).

Table 5.4 Best matches from NCBI gene database to nearly complete 16S rRNA gene sequences of isolates TD-01 and TD-02

	Match*	Gene	Query coverage (%)	Maximum Identity (%)
TD-01	<i>Shewanella haliotis</i> strain MS41	partial 16S rRNA gene	99	99
	Uncultured <i>Shewanella</i> sp. clone KSB6 16S ribosomal RNA gene, partial sequence	partial 16S rRNA gene	99	99
TD-02	<i>Ochrobactrum</i> sp. MVSV6	partial 16S rRNA gene	100	99
	<i>Ochrobactrum tritici</i>	partial 16S rRNA gene	100	99
	<i>Ochrobactrum</i> sp. CCBAU 10752	partial 16S rRNA gene	100	99

*All E-values were zero.

Shewanella strains are easily cultured when enrichment from environmental samples is followed by growth in general media [54]. Most *Shewanella* species have been cultured from marine environments [56, 57] and are usually implicated in the anaerobic spoilage of marine fish, and other protein-rich foods [58], perhaps because of its inability to utilize complex carbohydrates [59, 60]. Consistent with the characteristics of the genus, *S. haliotis* has been isolated from the gut of abalone (*Haliotidae*) [56]. *S. haliotis* is closely related to *S. alga*, one of the eight members of the *Shewanella* genus with a full genome sequenced. *S. alga* is also one of the few members detected in clinical samples [61, 62], able to grow at 37°C. Strain TD-01 grew to form visible pink colonies of 1 mm diameter from a 60-day culture on rich media plates at 37°C. *S. alga* and *S. haliotis* are both able to grow well at 37°C, in contrast to *S. oneidensis* MR-1, which grows optimally at 30°C and is unable to grow at 37°C.

Ochrobactrum colonies also grew in rich media within 24 hours (ASW-LB plates) and appeared as round, smooth white colonies.

Metal reduction ability of *S. haliotis* and *Ochrobactrum*

S. haliotis TD-01 and *Ochrobactrum* TD-02 were grown anaerobically with Mn(III) and Fe(III) citrate as electron acceptors and lactate as the electron donor in minimal media (M1). *S. haliotis* was able to reduce Mn(III) and Fe(III) citrate only in the presence of lactate or acetate, but not in the absence of a carbon source, at rates comparable to *S. oneidensis* MR-1 (Figure 5.8). *Ochrobactrum* strain TD-02 was unable to reduce Mn(III) or Fe(III)-citrate under the conditions tested. Similarly, in a previous study an *Ochrobactrum* sp. was isolated for the ability to reduce electrodes, but not metal

oxides, with acetate as electron donor [35]. Interestingly, TD-01 was also able to use acetate as an electron donor during anaerobic Mn(III) reduction (Figure 5.9). Previous studies have shown that *S. oneidensis* MR-1 only uses acetate as electron donor during aerobic conditions [63, 64], and a recent study showed that two members of the *Shewanella* genus, *S. lohica* and *S. denitrificans* are able to oxidize acetate anaerobically during denitrification, but not during reduction of Fe(III) [59]. Acetate utilization during metal reduction by strain TD-01 is unprecedented in the *Shewanella* genus and raises questions regarding the evolution of functional diversity of the *Shewanella* genus. During the first 3 generations, the enrichment strategy consisted of incubations with Mn(III)-pyrophosphate prepared from Mn(III)-acetate, which introduced acetate to the incubation media at a concentration of 200 μ M. Subsequently, acetate-free Mn(III)-pp was used. The initial inclusion of acetate in the incubation media may have selected for *Shewanella* strains capable of acetate utilization coupled to Mn(III) reduction that initially outcompeted strictly autotrophic strains of slower growth.

In summary, an enrichment culture amended with NH_4^+ and Mn(III) resulted in the selection of two strains belonging to the *Shewanella* and *Ochrobactrum* genus. *Shewanella* appears to be responsible for the Mn(III) reduction observed in the cultures, but the source of electron donors able to sustain the enrichment is still not conclusively determined. Further experimentation is required to show that *Shewanella* and *Ochrobactrum* are members of an ammonium-oxidizing, manganese-reducing consortia.

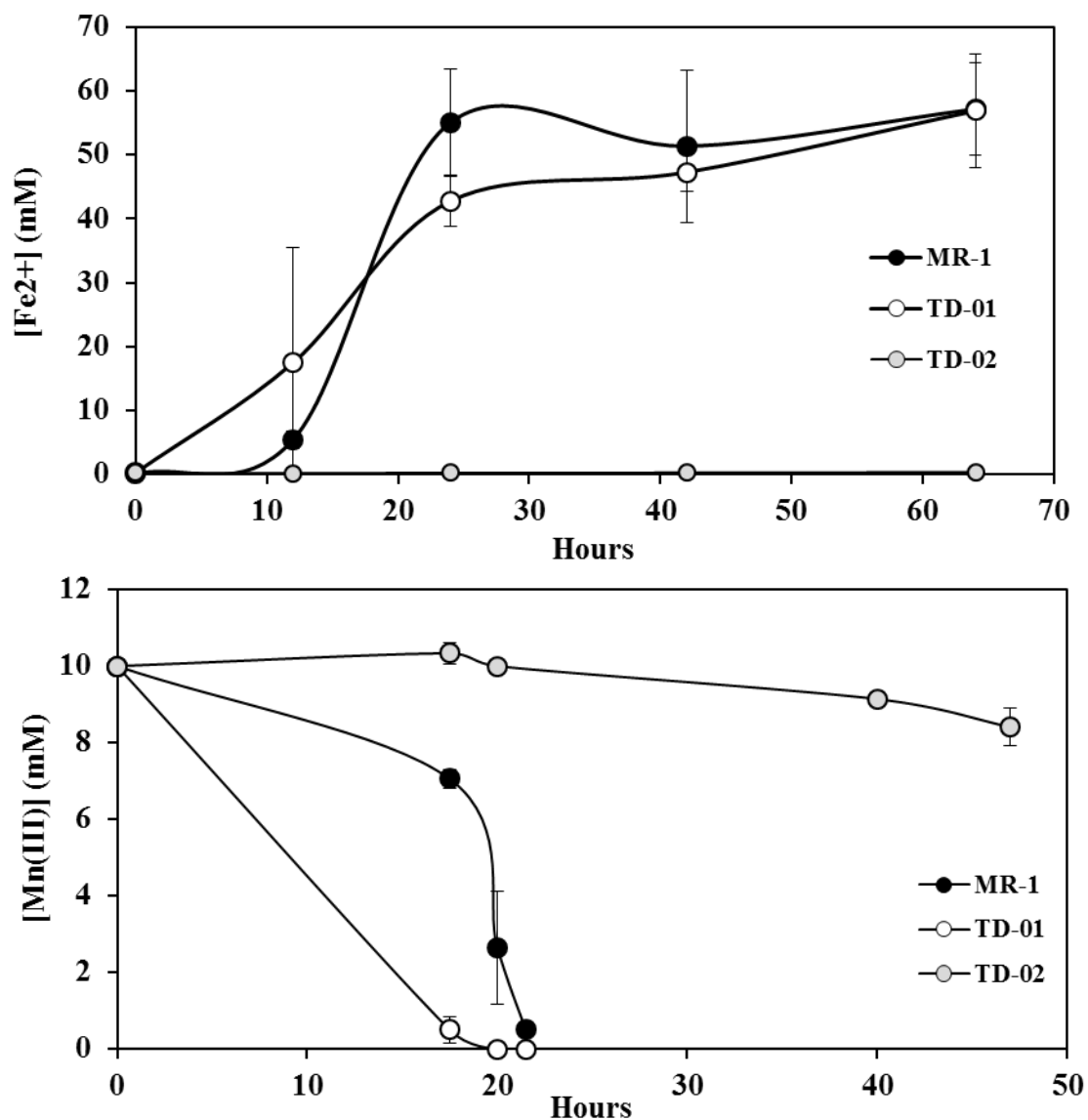


Figure 5.8. Rates of (A) Mn(III) and (B) Fe(III) reduction of the isolated strain TD-01 incubated under anaerobic conditions with lactate as electron donor. The results are the means of duplicate determinations, and standard deviations are shown as error bars.

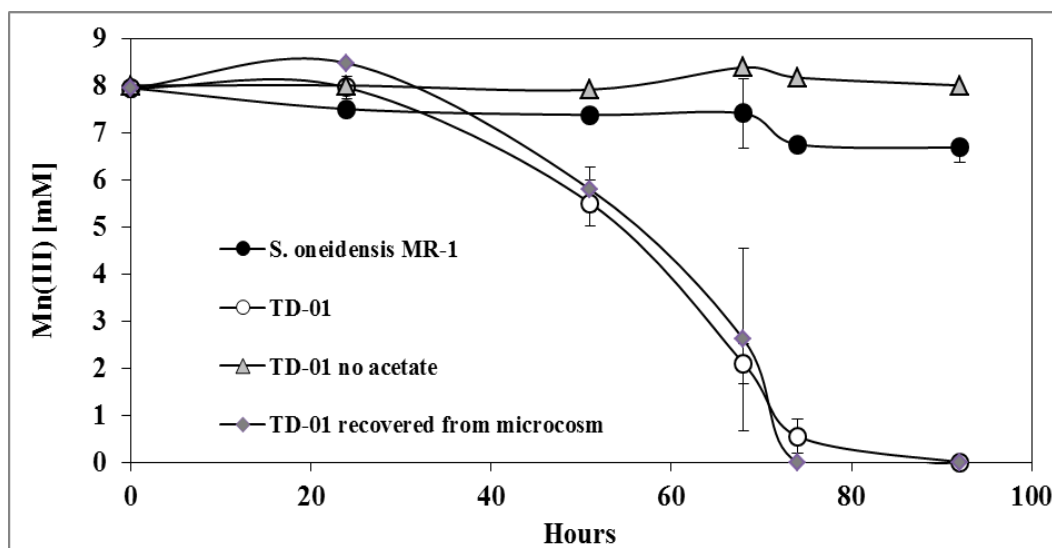


Figure 5.9. Rates of Mn(III) reduction of the isolated strain TD-01 incubated under anaerobic conditions with acetate as electron donor. The results are the means of duplicate determinations, and standard deviations are shown as error bars.

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Mn(IV) transformations play an important role in biogeochemical cycles of carbon and other elements. Microbial Mn(III) reduction is an important yet overlooked redox process in anaerobic marine and freshwater environments. The work of this dissertation investigated the molecular mechanism of microbial manganese respiration and the potential link between the Mn and N cycles in the form of three main hypotheses.

- 1) The mtr pathway, required for Mn(IV) and Fe(III) reduction, is also required for Mn(III) reduction in *S. onediensis*
- 2) Microbial Mn(IV) occurs via two one-electron transfer reactions
- 3) Microorganisms capable of NH_4^+ oxidation coupled to Mn(III) reduction can be selected in an enrichment culture with defined media

The current study revealed that 1) Mn(IV) reduction proceeds via two consecutive one-electron transfer reactions generating Mn(III) as a transient intermediate, that 2) the biochemical pathway for Mn(III) reduction in *S. oneidensis* MR-1 shares components of the Fe(III)- and Mn(IV)-reducing Mtr pathway, but OmcA may be a hydrogenase-dependant Mn(III) reduction-specific cytochrome, 3) *S. oneidensis* MR-1 requires other genes that are not part of the Mtr pathway, and are Mn(III) reduction-specific and 5) *S. haliotis* is able to reduce Mn(III) and Fe(III) and can use acetate during anaerobic respiration of Mn(III).

The metal reduction pathway for Fe(III) and Mn(IV) had been traditionally regarded as identical. The absence of OmcA has never been shown to significantly affect metal respiration rates in *S. oneidensis*, possibly because lactate has been the primary

electron donor tested in most studies. OmcA mutants grown with H₂ as electron donor, however, revealed a possible essential role of OmcA in Mn(III) reduction. OmcA seems to be dependent on hydrogenase activity, which produces H₂ during lactate and formate oxidation. H₂ may then diffuse through the membranes to reduce OmcA, which subsequently reduces Mn(III). The hypothesis could be tested by determining Mn(III) reduction rate differences during incubations with increasing H₂ concentrations. Increased reduction rates directly proportional to H₂ concentration in wild-type strains but not in OmcA deletion mutants would support the hypothesis.

Other authors have proposed that the mtr pathway is modular [1] based on an analysis of reduction rates of deleted combinations of mtrCAB and mtrDEF genes. Mtr pathway mutants showed wild-type Fe(III) reduction rates detected only during extended incubation periods. During Mn(IV) or Mn(III) reduction, however, the phenotype was not observed. It is possible that mtrDEF are expressed only during Fe(III) reduction. Quantifying mRNA of mtrDEF by qPCR on mtrCAB mutants may support the hypotheses that mtrDEF are involved in mtrCAB modularity but only during Fe(III) reduction.

Genomic analysis of the Mn(III) reduction-deficient mutant in Chapter 4 indicated that the mutated genes are not part of the Mtr pathway. The proteins encoded by these genes may be responsible for catalyzing the final electron transfer step, perhaps acting as a stabilizing ligand for Mn(III). Further exploration into the potential candidates is necessary. Point mutant Mn-2 is another interesting candidate, as it is able to reduce the extracellular electron acceptor DMSO but not Mn(III) or Fe(III). Further characterization

of respiratory flexibility in this mutant is needed to determine whether the mutation is metal-specific, and to subsequently identify the mutated gene.

The last objective of this thesis was to test the hypothesis that the interactions of the Mn and NH_4^+ biogeochemical cycles may be coupled by microorganism(s) able to grow autotrophically with NH_4^+ as electron donor and Mn(III) as electron acceptor. In this study, a liquid culture was enriched in two dominant populations, *Shewanella* and *Ochrobactrum*, that reduce Mn(III) anaerobically with NH_4^+ as the sole electron donor. The work of this thesis unveiled a second *Shewanella* strain able to reduce Mn(III) as electron acceptor. Mn(III) reduction, therefore, has been exclusively found in the *Shewanella* genus. Other metal and non-metal reducing strains should be tested for Mn(III) reduction. Other pathways different than the mtr pathway may be employed for extracellular reduction of Mn(III).

The enriched culture was unable to reduce Mn(III) in the absence of vitamins and still exhibited significant Mn(III) reduction rates and extent in the absence of NH_4^+ . Members of the *Shewanella* genus are considered to be heterotrophs, and no *Shewanella* species have yet been reported to fix inorganic carbon, nor have the genetic potential to do so [2, 3]. The ability of the *S. haliotis* isolate to reduce metals in the presence of organic carbon (lactate and acetate) and the lack of Mn(III) reduction activity of the enrichment in the absence of vitamins therefore suggests that Mn(III) reduction in the enriched culture incubations may be coupled to vitamin oxidation.

Vitamin availability, synthesis and consumption may control the distribution and abundance of bacterial populations in the ocean [4]. For example, vitamins are found at extremely low concentrations (often below detection levels) in the ocean, and their

limiting concentration is possibly a driver of bacterial community composition . Vitamins are normally required at picomolar concentrations and each one of them was added at 0.2 μM allowing members of the population in the enrichment culture to potentially use vitamins as carbon and energy sources. Bacterial populations under starvation conditions may employ the vitamins not as cofactors, but as carbon and nitrogen sources. Many of the vitamins added to the media have carboxylic groups, which can be oxidized anaerobically and potentially sustain growth. The N, S and C composition of the vitamins can be found in Table 5.2. This finding may also explain the slight increase in NH_4^+ observed in Figure 5.2. In addition, there are no studies that report the use of vitamins as carbon sources for the dominant populations that belong to the *Shewanella* and *Ochrobactrum* genus. Degradation products may be supporting a syntrophic interaction between these two species in the enrichment culture. Growth of *Shewanella* and *Ochrobactrum* strains isolated in this work with individual vitamins and testing and analysis of degradation products would provide more pieces of evidence to generate hypotheses for the interactions between these two organisms.

The success of *Shewanella* and other opportunitrophs may depend on the ease at which they enter and leave a dormant state and how fast they can be resuscitated by the addition of suitable nutrients. Only one study has focused on the practical implications for dormancy in *Shewanella* strains. In this study, *Shewanella* size changes as a function of nutrient load was studied to determine their transport capacity in soil pores during bioremediation of compounds in areas of the soil that are difficult to reach [5]. A large portion of the literature devoted to studies on *Shewanella* metal reduction ability, however, describes experiments based on growth in rich media and high cell densities.

Studies with more naturally relevant experimental conditions (low or fluctuating substrate concentration and cell densities of about 10^5) are also necessary to predict the role of *Shewanella* in the environment.

Finally, while this study did not provided only preliminary evidence of the potential role of NH_4^+ as electron donor during Mn(III) reduction, shown as a slight increase in Mn(III) reduction compared to the unamended control. Further testing is required to determine whether this redox couple exists in nature and supports growth. Sediment samples from other sites that show a similar profile of NH_4^+ depletion and Mn(II) production would increase the chances of finding organisms able to grow on NH_4^+ coupled to Mn. Employing lab-scale sequencing batch reactors as an enrichment strategy can also provide more biomass and more stable conditions than batch cultures to test hypotheses of substrate consumption and production.

The enrichment culture amended with NH_4^+ and Mn(III) resulted in selection for two dominant strains belonging to the *Shewanella* and *Ochrobactrum* genus. Both strains were recovered as pure colonies in rich media plates, and the *Shewanella* strain was identified as a *S. haliotis* strain. There are no genome sequences available for the two strains. Sequencing of the isolated strains would be useful to generate hypothesis about ammonia-oxidation potential, carbon fixation potential, metal reduction or extracellular respiration potential or vitamin degradation potential of each strain, as well as to generate species-specific primers to use during experiments in the lab (quantitative PCR). Further monitoring of gene abundance (or expression) with reverse-transcription can provide information about the successive role of both strains between the inoculation point and the maximum extent of Mn(II) reduction.

S. haliotis was able to reduce Mn(III) with lactate and acetate as electron donor, and Fe(III) with lactate as electron donor. Extracellular respiration on non-metal substrates such as DMSO should be studied to further understand the biochemical mechanism of extracellular respiration by this species. The Mn(III)- and Fe(III)-reducing abilities of *S. haliotis* have not been previously reported, and this study describes for the first time a member of the *Shewanella* genus able to use acetate as electron donor during anaerobic metal reduction. *S. oneidensis* is a metal reducer unable to use acetate anaerobically, while *S. loihica* can use acetate anaerobically but does not reduce metals. The differences between these strains raise interesting questions about the evolution of pathways that couple oxidation of organic carbon to metal reduction. Genomic comparison between *S. oneidensis*, *S. loihica* and *S. haliotis* genomes and proteomes may reveal if the metabolic differences are a product of the presence or absence of the genes required for acetate oxidation or if the differences are due to gene regulation, which could be further tested with qPCR. Genes targeted may include isocitrate lyase and malate synthase, components of the glyoxylate pathway.

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PROTEIN CALIBRATION CURVES

Protein calibration curves corrected for Mn concentration.

The Bradford reagent (Pierce, Waltham, MA) employed in this study was sensitive to the speciation and concentrations of Mn in solution (A). Mn(II) and Mn(III) over- and under-estimated the standard protein concentration (BSA), respectively. Increasing Mn(II) concentrations also increased the BSA protein values artificially (B) and increasing Mn(III) concentrations, reduced it (C). Protein measures in the enrichment incubations were therefore carried out by reducing the 200 μ M Mn(III) to Mn(II) by addition of sodium dithionite (1mM), which did not affect the reagent (D).

